Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/043696

International filing date: 23 December 2004 (23.12.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/532,004
Filing date: 23 December 2003 (23.12.2003)

Date of receipt at the International Bureau: 11 February 2005 (11.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

12498-47

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

February 03, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: ***60/532,004***
FILING DATE: ***December 23, 2003***
RELATED PCT APPLICATION NUMBER: ***PCT/US04/43696***

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
ATTENTION: PROVISIONAL PATENT APPLICATION

Sir:

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR § 1.53(c).

For: **DEFINITIVE ENDODERM**

Name of First Inventor: Emmanuel E. Baetge
Residence Address: 308 Sunset Drive, Encinitas, CA 92024

Name of Second Inventor: Kevin Allen D'Amour
Residence Address: 7235 Caminito Pantoja, San Diego, CA 92122

Name of Third Inventor: Alan D. Agulnick
Residence Address: 1259 Stratford Ct., San Marcos, CA 92069

Enclosed are:

(X) Specification in 47 pages.
(X) 37 sheets of drawings.
(X) A check in the amount of $80 to cover the filing fee is enclosed.
(X) A return prepaid postcard.
(X) The Commissioner is hereby authorized to charge any additional fees which may be required, now or in the future, or credit any overpayment to Account No. 11-1410.

Was this invention made by an agency of the United States Government or under a contract with an agency of the United States Government?

(X) No.

(X) Please send correspondence to:

Jerry L. Hefner
Knobbe, Martens, Olson & Bear, LLP
2040 Main Street, 14th Floor
Irvine, CA 92614

Respectfully submitted,

Jerry L. Hefner
Registration No. 53,009
Customer No. 20,995
(619) 235-8550

S:\DOCS\JLH\JLH-2452.DOC\122303

Knobbe Martens Olson & Bear LLP
Intellectual Property Law

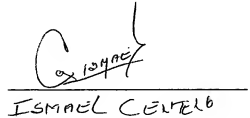
2040 Main Street
Fourteenth Floor
Irvine, CA 92614
Tel 949-760-0404
Fax 949-760-9502
www.kmob.com

MAIL STOP PROVISIONAL PATENT APPLICATION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

Attorney Docket No.	:	CYTHERA.039PR
Applicant(s)	:	Baetge et al.
For	:	DEFINITIVE ENDODERM
Attorney	:	Jerry L. Hefner
"Express Mail" Mailing Label No.	:	EV 323832257
Date of Deposit	:	December 23, 2003

I hereby certify that the accompanying transmittal letter; specification in 47 pages; 37 sheets of drawings; check for filing fee; return prepaid postcard are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and are addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.



ISMAEL CENTELLO

S:\DOCS\JLH\JLH-2451.DOC\122303

DEFINITIVE ENDODERM

Field of the Invention

[0001] The present invention relates to the fields of medicine and cell biology. In particular, the present invention relates to compositions comprising definitive endoderm cells and methods of making and using these cells.

Background

[0002] Human embryonic stem (ES) cells or human embryonic germ (EG) cells were first isolated in culture without fibroblast feeders in 1994 (Bongso et al., 1994) and with fibroblast feeders (Hogan, 1997; Labosky et al., 1994a; Labosky et al., 1994b). Later, Thomson, Reubinoff and Shamblott established continuous cultures of human ES and EG cells using mitotically inactivated mouse feeder layers (Reubinoff et al., 2000; Shamblott et al., 1998; Thomson et al., 1998).

[0003] Human ES and EG cells (hESCs) offer unique opportunities for investigating early stages of human development as well as for therapeutic intervention in several disease states, such as diabetes mellitus and Parkinson's disease. For example, the use of insulin-producing β-cells derived from hESCs would offer a vast improvement over current cell therapy procedures which utilize cells from donor pancreases. Currently cell therapy treatments for diabetes mellitus, which utilize cells from donor pancreases, are limited by the scarcity of high quality islet cells needed for transplant. Cell therapy for a single Type I diabetic patient requires a transplant of approximately 8×10^8 pancreatic islet cells. (Shapiro et al., 2000; Shapiro et al., 2001a; Shapiro et al., 2001b). As such, at least two healthy donor organs are required for to obtain sufficient islet cells for a successful transplant. HESCs offer a source of starting material from which to develop substantial quantities of high quality differentiated cells for human cell therapies.

[0004] Two properties that make hESCs uniquely suited to cell therapy applications are pluripotence and the ability to culture for prolonged periods without accumulation of genetic changes. Pluripotence is defined by the ability of hESCs to differentiate to derivatives of all 3 primary germ layers (endoderm, mesoderm, ectoderm)

which, in turn, form all cell types of the mature organism. Although pluripotency imparts extraordinary utility upon hESCs, this property also poses unique challenges for the study and manipulation of these cells and their derivatives. Owing to the large variety of cell types that may arise in differentiating hESC cultures, the vast majority of cell types are produced at very low efficiencies. Additionally, success in evaluating production of any given cell type depends critically on defining appropriate markers. Achieving efficient, directed differentiation is of great importance for therapeutic application of hESCs.

[0005] In order to use hESCs in cell therapy, it would be useful to overcome the foregoing problems. For example, in order to achieve the level of cellular material required for islet cell transplant therapy, it would be useful to efficiently direct hESCs toward the pancreatic islet/ β-cell lineage at the very earliest stages of differentiation.

Summary of the Invention

[0006] One embodiment of the present invention relates to novel, defined processes for the production of definitive endoderm cells in culture using stem cells, such as hESCs. These processes provide the basis for efficient production of endodermal derived tissues such as pancreas, liver, lung, stomach, intestine and thyroid. For example, production of definitive endoderm may be the first step in differentiation of the stem cell to a functional insulin-producing β-cell. Although high efficiency of differentiation is useful for all of the many fate decisions that occur prior to reaching the pancreatic islet/ β-cell fate as shown in Figure 1, high efficiency differentiation of stem cells to definitive endoderm cells is important because it represents an early step towards the production of functional pancreatic islet/beta cells.

[0007] One embodiment of the present invention is to define the process by which definitive endoderm is first formed and to be able to consistently produce enriched populations of definitive endoderm cells and or tissues *in vitro* from embryonic stem cells. Some aspects relate to *in vitro* methodology for the production of definitive endoderm from human embryonic stem cells. For example, one method encompasses the application of culture and growth factor conditions in a defined and temporally specified fashion resulting in a 20-100-fold enrichment in definitive endoderm cells versus no treatment at all.

[0008] In some embodiments of the present invention, the amount of definitive endoderm cells present in a culture can be determined by SOX17 Q-PCR analysis coupled with immunohistochemistry for Sox17 protein and by the partial exclusion of ectoderm, mesoderm and extra-embryonic endoderm fates as determined by gene specific Q-PCR and immunohistochemistry. In some embodiments, the stem cell to definitive endoderm differentiation procedure results in a 50-80% conversion of undifferentiated human stem cells to definitive endoderm cells coupled with greater than 2 orders of magnitude increase in SOX17 gene expression. In other embodiments, conversion of a stem cell population to substantially pure definitive endoderm cell population is contemplated.

[0009] Some embodiments of the present invention relate to compositions which comprise both stem cells and definitive endoderm cells. In some embodiments the number of stem cells is greater than the number of definitive endoderm cells, whereas in other embodiments, the number of definitive endoderm cells is greater than the number of stem cells. In some embodiments, the number of stem cells and definitive endoderm cells is approximately equivalent.

[0010] Other embodiments of the present invention include compositions comprising stem cells, definitive endoderm cells and one or more growth factors. In some embodiments, the one or more growth factors comprise the Nodal/Activin and BMP subgroups of the TGFβ superfamily of growth factors. In some embodiments, the one or more growth factors are selected from the group consisting of Nodal, Activin A, Activin B, BMP4, Wnt3a or combinations of any of these growth factors.

[0011] Still other embodiments of the present invention relate to substantially purified definitive endoderm cells. In some embodiments, the definitive endoderm cells express the SOX17 gene to a greater extent than the SOX7, the AFP and/or the SPARC genes.

[0012] Additional embodiments of the present invention are in accordance with the following numbered paragraphs:

[0013] 1. A composition comprising stem cells and definitive endoderm cells, wherein at least 5 definitive endoderm cells are present for about every 95 stem cells present in said composition.

[0014] 2. The composition of Paragraph 1, wherein at least 10 definitive endoderm cells are present for about every 90 stem cells present in said composition.

[0015] 3. The composition of Paragraph 1, wherein at least 20 definitive endoderm cells are present for about every 80 stem cells present in said composition.

[0016] 4. The composition of Paragraph 1, wherein at least 30 definitive endoderm cells are present for about every 70 stem cells present in said composition.

[0017] 5. The composition of Paragraph 1, wherein at least 40 definitive endoderm cells are present for about every 60 stem cells present in said composition.

[0018] 6. The composition of Paragraph 1, wherein at least 50 definitive endoderm cells are present for about every 50 stem cells present in said composition.

[0019] 7. The composition of Paragraph 1, wherein at least 60 definitive endoderm cells are present for about every 40 stem cells present in said composition.

[0020] 8. The composition of Paragraph 1, wherein at least 70 definitive endoderm cells are present for about every 30 stem cells present in said composition.

[0021] 9. The composition of Paragraph 1, wherein at least 80 definitive endoderm cells are present for about every 20 stem cells present in said composition.

[0022] 10. The composition of Paragraph 1, wherein at least 90 definitive endoderm cells are present for about every 10 stem cells present in said composition.

[0023] 11. The composition of Paragraph 1, wherein at least 95 definitive endoderm cells are present for about every 5 stem cells present in said composition.

[0024] 12. The composition of Paragraph 1, wherein said stem cells are embryonic stem cells.

[0025] 13. The composition of Paragraph 12, wherein said embryonic stem cells are derived from the inner cell mass of an embryo.

14. The composition of Paragraph 12, wherein said embryonic stem cells are derived from the gonadal ridges of an embryo.

[0026] 15. The composition of Paragraph 1, wherein said stem cells are of human origin.

[0027] 16. The composition of Paragraph 1, wherein said definitive endoderm cells are of human origin.

[0028] 17. The composition of Paragraph 1, wherein said definitive endoderm cells express the SOX17 gene.

[0029] 18. The composition of Paragraph 17, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the SOX7 gene.

[0030] 19. The composition of Paragraph 18, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the AFP gene.

[0031] 20. The composition of Paragraph 19, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the SPARC gene.

[0032] 21. The composition of Paragraph 20, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the Thrombomodulin gene.

[0033] 22. The composition of Paragraph 21, wherein said definitive endoderm cells express the MIXL1 gene.

[0034] 23. The composition of Paragraph 21, wherein said definitive endoderm cells express the GATA4 gene.

[0035] 24. The composition of Paragraph 21, wherein said definitive endoderm cells express the HNF3b gene.

[0036] 25. The composition of Paragraph 1 further comprising a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily.

[0037] 26. The composition of Paragraph 1 further comprising a growth factor of the BMP subgroup of the TGFβ superfamily.

[0038] 27. The composition of Paragraph 1 further comprising a growth factor selected from the group consisting of Nodal, Activin A, Activin B, BMP4, Wnt3a and combinations thereof.

[0039] 28. The composition of Paragraph 1 further comprising Nodal, Activin A, Activin B and BMP4.

[0040] 29. A substantially purified definitive endoderm cell.

[0041] 30. The definitive endoderm cell of Paragraph 29, wherein said cell is derived from an embryonic stem cell.

[0042] 31. The definitive endoderm cell of Paragraph 29, wherein said cell expresses the SOX17 gene.

[0043] 32. The definitive endoderm cell of Paragraph 31, wherein the expression of the SOX17 gene is greater than the expression of the SOX7 gene.

[0044] 33. The definitive endoderm cell of Paragraph 32, wherein the expression of the SOX17 gene is greater than the expression of the AFP gene.

[0045] 34. The definitive endoderm cell of Paragraph 33 wherein the expression of the SOX17 gene is greater than the expression of the SPARC gene.

[0046] 35. The definitive endoderm cell of Paragraph 34, wherein the expression of the SOX17 gene is greater than the expression of the Thrombomodulin gene.

[0047] 36. The definitive endoderm cell of Paragraph 35, wherein said cell expresses the MIXL1 gene.

[0048] 37. The definitive endoderm cell of Paragraph 35, wherein said cell expresses the GATA4 gene.

[0049] 38. The definitive endoderm cell of Paragraph 35, wherein said cell expresses the HNF3b gene.

[0050] 39. The definitive endoderm cell of Paragraph 29, wherein said cell is of human origin.

[0051] 40. A method for producing definitive endoderm cells, said method comprising obtaining a stem cell culture; and providing to said culture a growth factor of the TGFβ superfamily in an amount sufficient to promote differentiation of at least a portion of said stem cell culture to definitive endoderm cells.

[0052] 41. The method of Paragraph 40, wherein at least 5 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

[0053] 42. The method of Paragraph 40, wherein at least 10 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

[0054] 43. The method of Paragraph 40, wherein at least 20 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

[0055] 44. The method of Paragraph 40, wherein at least 30 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

[0056] 45. The method of Paragraph 40, wherein at least 40 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

[0057] 46. The method of Paragraph 40, wherein at least 50 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

[0058] 47. The method of Paragraph 40, wherein at least 60 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

[0059] 48. The method of Paragraph 40, wherein at least 70 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

[0060] 49. The method of Paragraph 40, wherein at least 80 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

[0061] 50. The method of Paragraph 40, wherein at least 90 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

[0062] 51. The method of Paragraph 40, wherein at least 95 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

[0063] 52. The method of Paragraph 40, wherein the stem cells in said culture are embryonic stem cells.

[0064] 53. The method of Paragraph 52, wherein said embryonic stem cells are derived from the inner cell mass of an embryo.

[0065] 54. The method of Paragraph 52, wherein said embryonic stem cells are derived from the gonadal ridges of an embryo.

[0066] 55. The method of Paragraph 40, wherein said stem cells in said culture are of human origin.

[0067] 56. The method of Paragraph 40, wherein said definitive endoderm cells are of human origin.

[0068] 57. The method of Paragraph 40, wherein said definitive endoderm cells express the SOX17 gene.

[0069] 58. The method of Paragraph 57, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the SOX7 gene.

[0070] 59. The method of Paragraph 58, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the AFP gene.

[0071] 60. The method of Paragraph 59, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the SPARC gene.

[0072] 61. The method of Paragraph 60, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the Thrombomodulin gene.

[0073] 62. The method of Paragraph 61, wherein said definitive endoderm cells express the MIXL1 gene.

[0074] 63. The method of Paragraph 61, wherein said definitive endoderm cells express the GATA4 gene.

[0075] 64. The method of Paragraph 61, wherein said definitive endoderm cells express the HNF3b gene.

[0076] 65. The method of Paragraph 40, wherein said growth factor is of the Nodal/Activin subgroup of the TGFβ superfamily.

[0077] 66. The method of Paragraph 40, wherein said growth factor is of the BMP subgroup of the TGFβ superfamily.

[0078] 67. The method of Paragraph 40, wherein said growth factor is selected from the group consisting of Nodal, Activin A, Activin B, BMP4 and combinations thereof.

[0079] 68. The method of Paragraph 67 further comprising the growth factor Wnt3a.

[0080] 69. The method of Paragraph 40, wherein a plurality of growth factors of the TGFβ superfamily is provided.

[0081] 70. The method of Paragraph 69, wherein the plurality of growth factors comprises Nodal, Activin A, Activin B and BMP4.

[0082] 71. The method of Paragraph 40, wherein said growth factor is BMP4.

[0083] 72. The method of Paragraph 71 further comprising the step of removing BMP4 within approximately 4 days after its addition.

[0084] 73. The method of Paragraph 40 wherein said growth factor is provided in a concentration of at least 10 ng/ml.

[0085] 74. The method of Paragraph 40, wherein said growth factor is provided in a concentration of at least 25 ng/ml.

[0086] 75. The method of Paragraph 40, wherein said growth factor is provided in a concentration of at least 50 ng/ml.

[0087] 76. The method of Paragraph 40, wherein said growth factor is provided in a concentration of at least 75 ng/ml.

[0088] 77. The method of Paragraph 40, wherein said growth factor is provided in a concentration of at least 100 ng/ml.

[0089] 78. The method of Paragraph 40, wherein said growth factor is provided in a concentration of at least 200 ng/ml.

[0090] 79. The method of Paragraph 40, wherein said growth factor is provided in a concentration of at least 300 ng/ml.

[0091] 80. The method of Paragraph 40, wherein said growth factor is provided in a concentration of at least 400 ng/ml.

[0092] 81. The method of Paragraph 40, wherein said growth factor is provided in a concentration of at least 500 ng/ml.

[0093] 82. The method of Paragraph 40, wherein said growth factor is provided in a concentration of at least 1000 ng/ml.

[0094] 83. A definitive endoderm cell produced by the method of Paragraph 40.

[0095] 84. An antibody which binds to SOX17.

[0096] 85. The antibody of Paragraph 84, wherein said SOX17 is human SOX17.

[0097] 86. The antibody of Paragraph 84, wherein said antibody is a polyclonal.

[0098] 87. The antibody of Paragraph 84, wherein said antibody is a monoclonal antibody.

[0099] 88. A method of increasing the expression of the SOX17 gene product in a stem cell comprising contacting said stem cell with a growth factor of the TGFβ superfamily in an amount sufficient to increase expression of the SOX17 gene product.

[0100] 89. The method of Paragraph 88, wherein the stem cell is an embryonic stem cell.

[0101] 90. The method of Paragraph 89, wherein said embryonic stem cell is derived from the inner cell mass of an embryo.

[0102] 91. The method of Paragraph 89, wherein said embryonic stem cell is derived from the gonadal ridges of an embryo.

[0103] 92. The method of Paragraph 88, wherein said stem cell is of human origin.

[0104] 93. The method of Paragraph 88, wherein said growth factor is selected from the group consisting of Nodal, Activin A, Activin B, BMP4 and combinations thereof.

[0105] 94. The method of Paragraph 93 further comprising the growth factor Wnt3a.

[0106] 95. The method of Paragraph 88, wherein a plurality of growth factors of the TGFβ superfamily is provided.

[0107] 96. The method of Paragraph 95, wherein the plurality of growth factors comprises Nodal, Activin A, Activin B and BMP4.

[0108] 97. The method of Paragraph 88, wherein said growth factor is BMP4.

[0109] 98. The method of Paragraph 97 further comprising the step of removing BMP4 within approximately 4 days after its addition.

Brief Description of the Drawings

[0110] Figure 1 is a schematic of a proposed differentiation pathway for the production of beta-cells from hESCs. The first step in the pathway commits the ES cell to the definitive endoderm lineage and represents an essential step prior to further differentiation events to pancreatic endoderm, endocrine endoderm, or islet/beta-cell. Some factors useful for mediating this transition are highlighted in red. Relevant markers for defining the definitive endoderm target cell are listed in blue.

[0111] Figure 2A is a micrograph showing characteristic morphology of an undifferentiated hESC colony.

[0112] Figure 2B is an inset from Figure 2A showing the characteristic morphology (round with prominent nucleoli) of individual hESCs at 200X magnification.

[0113] Figure 3 is an image of the karyotype of hESCyT-25 at passage 10 and 28. hESCyT-25 displays a normal female karyotype that remains stable over time through passages 10 and 28.

[0114] Figure 4A-C are micrographs showing that hESCyT-25 cells display immunoreactivity for the characteristic markers of undifferentiated hESCs. (A) TRA-1-60, (B) OCT4, (C) SSEA4.

[0115] Figure 5A-D are micrographs which demonstrate that undifferentiated hESCyT-25 cells are recognizable by bright field morphology of colonies (A) and individual cells (C). It is also demonstrated that these cells display the hESC characteristic of robust alkaline phosphatase activity both in colonies (B) and individual cells (D).

[0116] Figure 6 is a micrograph which demonstrates that hESCyT-25 cells can be differentiated as floating embryoid bodies when cultured on low adherent substrata.

[0117] Figure 7 is a micrograph which shows the differentiation of hESCyT-25 to neurons (ectoderm germ layer) as indicated by immunoreactivity to βIII-tubulin.

[0118] Figure 8A is a line graph showing differentiation of hESCyT-25 to endoderm germ layer is indicated by the robust expression of SOX7 and AFP.

[0119] Figure 8B is a micrograph showing cell differentiated in monolayer which displays regions of strong immunoreactivity for AFP.

[0120] Figure 9 is a bar chart showing rapid increase in expression of brachyury with time under differentiating suspension culture conditions. Increased expression of brachyury is indicative of differentiation of hESCyT-25 to cells of mesoderm lineage.

[0121] Figure 10 is a diagram of the human SOX17 cDNA which displays the positions of conserved motifs and highlights the region used for the immunization procedure by GENOVAC.

[0122] Figure 11 is a Western blot probed with the rat anti-SOX17 antibody. This blot demonstrates the specificity of this antibody for human SOX17 protein over-expressed in fibroblasts (lane 1) and a lack of immunoreactivity with EGFP (lane 2) or the most closely related SOX family member, SOX7 (lane 3).

[0123] Figure 12A-F are micrographs which demonstrate SOX17 antibody specificity by immunocytochemistry. Panels A-C show fibroblasts which were transfected

with human SOX17 and EGFP cDNA whereas panels D-F show fibroblasts transfected with EGFP alone. SOX17 immunoreactive cells (red) are observed only in SOX17 transfected cultures (A and C but not D and F) and reactivity correlates with the EGFP signal (green – B and E) indicating that those cells were transfected (C).

[0124] Figure 13 is a relational dendrogram illustrating that SOX17 is most closely related to SOX7 and somewhat less to SOX18. The SOX17 proteins are more closely related among species homologs than to other members of the SOX group F subfamily within the same species.

[0125] Figure 14A-B are micrographs showing a cluster of $SOX17^+$ (red) cells that display a significant number of AFP^+ (green) co-labeled cells (A). This is in striking contrast to other $SOX17^+$ clusters (B) where little or no AFP^+ cells are observed.

[0126] Figure 15A-C are micrographs showing parietal endoderm and SOX17. Panel A shows immunocytochemistry (green fluorescence) for human Thrombomodulin (TM) protein located on the cell surface of parietal endoderm cells in randomly differentiated cultures of hES cells. Panel B is the identical field shown in A that is double-labeled for TM (green) and Sox17 (red). Panel C is the phase contrast image of the same field with DAPI labeled nuclei. Note the complete correlation of DAPI labeled nuclei and Sox17 labeling.

[0127] Figure 16A-B are bar charts showing Sox17 gene expression by quantitative PCR (Q-PCR) and anti-Sox17 positive cells by Sox17-specific antibody. Panel A shows that Activin A increases SOX17 gene expression while retinoic acid (RA) strongly suppresses SOX17 expression relative to the undifferentiated control media (SR20). Panel B shows the identical pattern as well as a similar magnitude of these changes is reflected in $SOX17^+$ cell number, indicating that Q-PCR measurement of SOX17 gene expression is very reflective of changes at the single cell level.

[0128] Figure 17A is a bar charts which shows that a culture of differentiating hESCs in the presence of Activin A maintains a low level of AFP gene expression while cells allowed to randomly differentiate in 10% fetal bovine serum (FBS) exhibit a strong upregulation of AFP. The difference in expression levels is approximately 7-fold.

[0129] Figure 17B is an image of two micrographs showing that the suppression of AFP expression by Activin A is also evident at the single cell level as indicated by the very

rare and small clusters of AFP^+ cells observed in Activin A treatment conditions (bottom) relative to 10% FBS alone (top).

[0130] Figure 18 is a comparative image showing the quantitation of the AFP^+ cell number using flow cytometry. This figure demonstrates that the magnitude of change in AFP gene expression (Figure 17A) in the presence (right panel) and absence (left panel) of Activin A exactly corresponds to the number of AFP^+ cells, further supporting the utility of Q-PCR analyses to indicate changes occurring at the individual cell level.

[0131] Figure 19A-F are micrographs which show that exposure of hESCs to nodal, Activin A and Activin B (NAA) yields a striking increase in the number of $SOX17^+$ cells over the period of 5 days (A-C). By comparing to the relative abundance of $SOX17^+$ cells to the total number of cells present in each field, as indicated by blue DAPI stained nuclei (D-F), it can be seen that approximately 30-50% of all cells are immunoreactive for SOX17 after five days treatment with NAA.

[0132] Figure 20 is an image displaying graphs that are representative examples of the 3-point standard curve and melt curve analysis using the Rotor Gene 3000 instrument for Q-PCR. Prerequisites for acceptable Q-PCR performance are high correlation coefficients (≥ 0.98), PCR efficiency values near 100%, and the presence of a single PCR product with no amplification from samples that did not receive reverse transcriptase.

[0133] Figure 21 is a bar chart which demonstrates that Activin A (0, 10, 30 or 100 ng/mL) dose-dependently increases SOX17 gene expression in differentiating hESCs. Increased expression is already robust after 3 days of treatment on adherent cultures and continues through subsequent 1, 3 and 5 days of suspension culture as well.

[0134] Figure 22A-C are bar charts which demonstrate the effect of Activin A on the expression of MIXL1 (panel A), GATA4 (panel B) and HNF3b (panel C). Activin A dose-dependent increases are also observed for three other markers of definitive endoderm; MIXL1, GATA4 and HNF3b. The magnitudes of increased expression in response to activin dose are strikingly similar to those observed for SOX17, strongly indicating that Activin A is specifying a population of cells that co-express all four genes ($SOX17^+$, $MIXL1^+$, $GATA4^+$ and $HNF3b^+$).

[0135] Figure 23A-C are bar charts which demonstrate the effect of Activin A on the expression of AFP (panel A), SOX7 (panel B) and SPARC (panel C). There is an Activin A dose-dependent decrease in expression of the visceral endoderm marker AFP. Markers of primitive endoderm (SOX7) and parietal endoderm (SPARC) remain either unchanged or exhibit suppression at some time points indicating that Activin A does not act to specify these extra-embryonic endoderm cell types. This further supports the fact that the increased expression of SOX17, MIXL1, GATA4, and HNF3b are due to an increase in the number of definitive endoderm cells in response to Activin A.

[0136] Figure 24A-B are bar charts showing the effect of Activin A on ZIC1 (panel A) and Brachyury expression (panel B) Consistent expression of the neural marker ZIC1 demonstrates that there is no dose-dependent effect of Activin A on neural differentiation. There is a notable suppression of mesoderm differentiation mediated by 100 ng/mL of Activin A treatment as indicated by the decreased expression of brachyury. This is likely the result of the increased specification of definitive endoderm from the mesendoderm precursors. Lower levels of Activin A treatment (10 and 30 ng/mL) maintain the expression of brachyury at later time points of differentiation relative to untreated control cultures.

[0137] Figure 25A-C are micrographs showing (A) hESCs at the periphery of the colony exhibit signs of differentiation indicated by their elongated morphologies (arrows). (B, C) These same cells also appear to have increased immunoreactivity for OCT4.

[0138] Figure 26A-F are micrographs showing that undifferentiated hESCs expressing OCT4 do not express SOX17. In hESC colonies of primarily undifferentiated phenotype (A, C, E), $SOX17^{+}$ cells occur only at the periphery where differentiated cells begin to appear and none co-label with OCT4 which is found throughout the remainder of the colony (C). In colonies where differentiation is widespread (B, D, F) clusters $SOX17^{+}$ cells are present scattered throughout the interior of the colony as well as at the edges and boundaries between colonies (D). However, even in colonies of such heterogenous state, OCT4 and SOX17 immunoreactivity is not found in the same cells although they occur highly intermingled with each other. (OCT4 – red, SOX17 – green, DAPI – blue).

[0139] Figure 27A-B are micrographs showing decreased parietal endoderm differentiation in response to treatment with activins. Regions of TM^{hi} parietal endoderm are

found through the culture (A) when differentiated in serum alone, while differentiation to TM^+ cells is scarce when activins are included (B) and overall intensity of TM immunoreactivity is lower.

[0140] Figure 28 is a micrograph showing the appearance of definitive endoderm and visceral endoderm in vitro from hESCs. The regions of visceral endoderm are identified by $AFP^{hi}/SOX17^{lo/-}$ while definitive endoderm displays the complete opposite profile, $SOX17^{hi}/AFP^{lo/-}$. It is possible that a small proportion of these $SOX17^{hi}/AFP^{lo/-}$cells may be parietal endoderm (see text). This field was selectively chosen due to the proximity of these two regions to each other. However, there are numerous times when $SOX17^{hi}/AFP^{lo/-}$ regions are observed in absolute isolation from any regions of AFP^{hi} cells, suggesting the separate origination of the definitive endoderm cells from visceral endoderm cells.

[0141] Figure 29 is diagram depicting the TGFβ family of ligands and receptors. Factors activating AR Smads and BR Smads are useful in the production of definitive endoderm from human embryonic stem cells (see, *J Cell Physiol.* **187**:265-76).

[0142] Figure 30 is a bar chart showing the induction of SOX17 expression over time as a result of treatment with individual and combinations of TGFβ factors.

[0143] Figure 31 is a bar chart showing the increase in $SOX17^+$ cell number with time as a result of treatment with combinations of TGFβ factors.

[0144] Figure 32 is a bar chart showing induction of SOX17 expression over time as a result of treatment with combinations of TGFβ factors.

[0145] Figure 33 is a bar chart showing that Activin A induces a dose-dependent increase in $SOX17^+$ cell number.

[0146] Figure 34 is a bar chart showing that addition of Wnt3a to Activin A and Activin B treated cultures increases SOX17 expression above the levels induced by Activin A and Activin B alone.

[0147] Figure 35A-C are bar charts showing differentiation to definitive endoderm is enhanced in low FBS conditions. Treatment of hESCs with activins A and B in media containing 2% FBS (2AA) yields a 2-3 times greater level of SOX17 expression as compared to the same treatment in 10% FBS media (10AA) (panel A). Induction of the definitive endoderm marker MIXL1 (panel B) is also affected in the same way and the

suppression of AFP (visceral endoderm) (panel C) is greater in 2% FBS than in 10% FBS conditions.

[0148] Figure 36A-D are micrographs which show $SOX17^+$ cells are dividing in culture. SOX17 immunoreactive cells (green) are present at the differentiating edge of an hESC colony (C, D) and are labeled with proliferating cell nuclear antigen (PCNA) (red in panel B) yet are not co-labeled with OCT4 (red in panel C). In addition, clear mitotic figures can be seen by DAPI labeling of nuclei in both $SOX17^+$ cells (arrows) as well as $OCT4^+$, undifferentiated hESCs (arrowheads) (D).

[0149] Figure 37A-D are micrographs which show hESC marker expression in response to treatment with Activin A and Activin B. hESCs were treated for four consecutive days with Activin A and Activin B and triple labeled with SOX17, AFP and TM antibodies. Panel A - SOX17; Panel B - AFP; Panel C - TM; and Panel D - Phase/DAPI. Notice the numerous SOX17 positive cells (A) associated with the complete absence of AFP (B) and TM (C) immunoreactivity.

Detailed Description

[0150] In accordance with some embodiments of the present invention, methods of producing definitive endoderm from stem cells, such as pluripotent stem cells, are disclosed. Stem cells used in these methods can include, but are not limited to, embryonic stem cells. Embryonic stem cells can be derived from the embryonic inner cell mass or from the embryonic gonadal ridges. Embryonic stem cells can originate from a variety of animal species including, but not limited to, various mammalian species including humans.

[0151] In some embodiments of the present invention, one or more growth factors are used in the differentiation process from stem cell to definitive endoderm cell. Such factors can include growth factors from the BMP subgroup of the TGFβ superfamily. For example, such factors include, but are not limited to Nodal, Activin A, Activin B, BMP4 and combinations thereof. The use of the growth factor Wnt3a is also contemplated.

[0152] Other aspects of the invention disclosed herein relate to compositions which comprise both stem cells and definitive endoderm cells. In some embodiments such

compositions also include one or more growth factors. In other embodiments, some compositions described herein are substantially purified definitive endoderm cells.

[0153] In some embodiments of the present invention SOX17 antibodies can be used to isolate definitive endoderm cells in a substantially purified form. Methods known in the art, such as affinity-based or magnetic-based separation, can be used to obtain substantially purified preparations of definitive endoderm cells bound to the SOX17 antibody.

[0154] The compositions and methods described herein have several useful features. For example, the compositions and methods described herein are useful for modeling the early stages of human development. Furthermore, the compositions and methods described herein can also serve for therapeutic intervention in disease states, such as diabetes mellitus. For example, since definitive endoderm serves as the source for only a limited number tissues, it can be used in the development of pure tissue or cell types.

[0155] A crucial stage in early human development termed gastrulation occurs 2-3 weeks after fertilization. Gastrulation is extremely significant because it is at this time that the three primary germ layers are first specified and organized (Lu et al., 2001; Schoenwolf and Smith, 2000). The ectoderm is responsible for the eventual formation of the outer coverings of the body and the entire nervous system whereas the heart, blood, bone, skeletal muscle and other connective tissues are derived from the mesoderm. Definitive endoderm is defined as the germ layer that is responsible for formation of the entire gut tube which includes the esophagus, stomach and small and large intestines, and the organs which derive from the gut tube such as the lungs, liver, thymus, parathyroid and thyroid glands, gall bladder and pancreas (Grapin-Botton and Melton, 2000; Kimelman and Griffin, 2000; Tremblay et al., 2000; Wells and Melton, 1999; Wells and Melton, 2000). A very important distinction should be made between the definitive endoderm and the completely separate lineage of cells termed primitive endoderm. The primitive endoderm is primarily responsible for formation of extra-embryonic tissues, mainly the parietal and visceral endoderm portions of the placental yolk sac and the extracellular matrix material of Reichert's membrane.

[0156] During gastrulation, the process of definitive endoderm formation begins with a cellular migration event in which mesendoderm cells (cells competent to form

mesoderm or endoderm) migrate through a structure called the primitive streak. Definitive endoderm is derived from cells, which migrate through the anterior portion of the streak and through the node (a specialized structure at the anterior-most region of the streak). As migration occurs, definitive endoderm populates first the most anterior gut tube and culminates with the formation of the posterior end of the gut tube.

[0157] *In vivo* analyses of the formation of definitive endoderm, such as the studies in Zebrafish and *Xenopus* by Conlon et al., 1994; Feldman et al., 1998; Zhou et al., 1993; Aoki et al., 2002; Dougan et al., 2003; Tremblay et al., 2000; Vincent et al., 2003; Alexander et al., 1999; Alexander and Stainier, 1999; Kikuchi et al., 2001; Hudson et al., 1997 and in mouse by Kanai-Azuma et al., 2002 lay a foundation for how one might attempt to approach the development of a specific germ layer cell type in the culture dish using human embryonic stem cells. There are two aspects associated with in vitro ESC culture that pose major obstacles in the attempt to recapitulate development in the culture dish. First, organized germ layer or organ structures are not produced. The majority of germ layer and organ specific genetic markers will be expressed in a heterogeneous fashion in the differentiating hESC culture system. Therefore it is difficult to evaluate formation of a specific tissue or cell type due to this lack of organ specific boundaries. Almost all genes expressed in one cell type within a particular germ layer or tissue type are expressed in other cells of different germ layer or tissue types as well. Without specific boundaries there is considerably less means to assign gene expression specificity with a small sample of 1-3 genes. Therefore one must examine considerably more genes, some of which should be present as well as some that should not be expressed in the particular cell type of the organ or tissue of interest. Second, the timing of gene expression patterns is crucial to movement down a specific developmental pathway.

[0158] To further complicate matters, it should be noted that stem cell differentiation *in vitro* is rather asynchronous, likely considerably more so than *in vivo*. As such, one group of cells may be expressing genes associated with gastrulation, while another group maybe starting final differentiation. Furthermore, manipulation of hESC monolayers or embryoid bodies (EBs) with or without exogenous factor application may result in profound differences with respect to overall gene expression pattern and state of

differentiation. For these reasons, the application of exogenous factors must be timed according to gene expression patterns within a heterogeneous cell mixture in order to efficiently move the culture down a specific differentiation pathway.

[0159] Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only, and are not intended to be limiting.

EXAMPLES

[0160] Many of the examples below describe the use of human embryonic stem cells. Methods of producing human embryonic stem cells are well known in the art and have been described numerous scientific publications.

EXAMPLE 1

Human ES cells

[0161] For our studies of endoderm development we employed human embryonic stem cells, which are pluripotent and can divide seemingly indefinitely in culture while maintaining a normal karyotype. ES cells were derived from the 5-day-old embryo inner cell mass using either immunological or mechanical methods for isolation. The hESC line designated hESCyT-25 was derived more than 18 months ago and has been serially passaged over 50 times. We employed the hESCyT-25 human embryonic stem cell line as our starting material for the production of definitive endoderm.

EXAMPLE 2

hESCyt-25 Characterization

[0162] The human embryonic stem cell line, hESCyt-25 has maintained a normal morphology, karyotype, growth and self-renewal properties over 18 months in culture as shown in Figures 2A-B and 3. hESCyt-25 displays strong immunoreactivity for the OCT4, SSEA-4 and TRA-1-60 antigens characteristic of undifferentiated hESCs (Figure 4A-C) and displays alkaline phosphatase activity as well as a morphology identical to other established hESC lines (Figure 5A-D). The hESCyT-25 cell line also readily forms embryoid bodies

when cultured in suspension as shown in Figure 6. As a demonstration of its pluripotential nature, hESCyT-25 differentiates into various cell types that represent the three principle germ layers. Ectoderm production is demonstrated by Q-PCR for ZIC1 as well as immunocytochemistry (ICC) for nestin and more mature neuronal markers. Immunocytochemical staining for β-III tubulin was observed in clusters of elongated cells, characteristic of early neurons (Figure 7). We treated EBs in suspension with retinoic acid, previously shown to induce differentiation of pluripotent stem cells to visceral endoderm (VE), an extra-embryonic lineage. Treated cells expressed high levels of α-fetoprotein (AFP) and SOX7, two markers of VE, by 54 hours of treatment (Figure 8A). Cells differentiated in monolayer expressed AFP in sporadic patches demonstrated by immunocytochemical staining as shown in Figure 8B. As will be extensively described below, definitive endoderm was formed, as validated by real-time quantitative polymerase chain reaction (Q-PCR) and immunocytochemistry for SOX17, in the absence of AFP expression. To demonstrate differentiation to mesoderm, differentiating EBs were analyzed for Brachyury gene expression at several time points. Brachyury expression increased progressively over the course of the experiment (Figure 9). Accordingly, the hESCyT-25 line is pluripotent as shown by the ability to form cells representing the three germ layers.

EXAMPLE 3

Production of Sox17 Antibody

[0163] A primary obstacle to the identification of definitive endoderm in hESC cultures is the lack of appropriate tools. We therefore undertook the production of an antibody raised against human SOX17 protein.

[0164] Sox17 is expressed throughout the definitive endoderm as it forms during gastrulation and its expression is maintained in the gut tube (although levels of expression vary along the A-P axis) until around the onset of organogenesis. Sox17 is also expressed in a subset of extra-embryonic endoderm cells. No expression of this protein has been observed in mesoderm or ectoderm. As such, Sox17 is an appropriate marker for the definitive endoderm lineage when used in conjunction with markers to exclude extra-embryonic lineages.

[0165] As described in detail herein, the SOX17 antibody was utilized to specifically examine effects of various treatments and differentiation procedures aimed at the production of SOX17 positive definitive endoderm cells. Other antibodies reactive to AFP, SPARC and Thrombomodulin were also employed to rule out the production of visceral and parietal endoderm (extra-embryonic endoderm).

[0166] The SOX17 antibody was produced by genetic immunization in rats according to procedures developed at the antibody production company GENOVAC (Freiberg, Germany). A portion of the human SOX17 cDNA corresponding to amino acids 172-414 in the carboxyterminal end of the SOX17 protein was used for immunization (Figure 10).

[0167] Suitable methods for genetic immunization are described, for example, in International Patent Application Publication No. WO00/29442 the disclosure of which is incorporated herein by reference in its entirety. Other methods of genetic immunization methodology are described, for example, in Barry, M.A., et al (1994) Production of monoclonal antibodies by genetic immunization. Biotechniques 16: 616-620; Specific examples of genetic immunization methods to produce antibodies against specific proteins can be found, for example, in Costaglia *et al.*, (1998) Genetic immunization against the human thyrotropin receptor causes thyroiditis and allows production of monoclonal antibodies recognizing the native receptor. *J. Immunol.* 160: 1458-1465; Kilpatrick *et al* (1998) Gene gun delivered DNA-based immunizations mediate rapid production of murine monoclonal antibodies to the Flt-3 receptor. *Hybridoma* 17: 569-576; Schmolke *et al.*, (1998) Identification of hepatitis G virus particles in human serum by E2-specific monoclonal antibodies generated by DNA immunization. *J. Virol.* 72: 4541-4545; Krasemann et al., (1999) Generation of monoclonal antibodies against proteins with an unconventional nucleic acid-based immunization strategy. *J. Biotechnol.* 73: 119-129; and Ulivieri *et al.*, (1996) Generation of a monoclonal antibody to a defined portion of the *Heliobacter pylori* vacuolating cytotoxin by DNA immunization. *J. Biotechnol.* 51: 191-194, the disclosures of which are incorporated herein by reference in their entireties.

[0168] The antibody was determined to be specific for SOX17 by both Western blot and ICC on hSOX17 cDNA transfected cell lines as shown in Figures 11 and 12. SOX7

and SOX18 are the closest Sox family relatives as depicted in the relational dendrogram shown in Figure 13. We employed human SOX7 as a negative control to demonstrate that the SOX17 antibody is absolutely specific for SOX17 and does not react with its closest family member.

[0169] The following methods were utilized for the production of the SOX17 and SOX7 expression vectors, their transfection into human fibroblasts and analysis by Western blot. Expression vectors employed for the production of SOX17, SOX7, and EGFP were pCMV6 (OriGene Technologies, Inc., Rockville, MD), pCMV-SPORT6 (Invitrogen, Carlsbad, CA) and pEGFP-N1 (Clonetech, Palo Alto, CA), respectively. For protein production, telomerase immortalized MDX human fibroblasts were transiently transfected with supercoiled DNA in the presence of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Total cellular lysates were collected 36 hours post-transfection in 50 mM TRIS-HCl (pH 8), 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, containing a cocktail of protease inhibitors (Roche Diagnostics Corporation, Indianapolis, IN). Western blot analysis of 100 µg of cellular proteins, separated by SDS-PAGE on NuPAGE (4-12 % gradient polyacrylamide, Invitrogen, Carlsbad, CA), and transferred by electro-blotting onto PDVF membranes (Hercules, CA), were probed with a 1/1000 dilution of the rat SOX17 anti-serum in 10 mM TRIS-HCl (pH 8), 150 mM NaCl, 10% BSA, 0.05 % Tween-20 (Sigma, St. Louis, MO), followed by Alkaline Phosphatase conjugated anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and revealed through Vector Black Alkaline Phosphatase staining (Vector Laboratories, Burlingame, CA). The proteins size standard used was wide range color markers (Sigma, St. Louis, MO).

[0170] In Figure 11, protein extracts made from human fibroblast cells that were transiently transfected with SOX17, SOX7 or EGFP cDNA's were probed on Western blots with the SOX17 antibody. Only the protein extract from hSOX17 transfected cells produced a band of ~51Kda which closely matches the predicted 46 Kda molecular weight of the human SOX17 protein. There was no reactivity of the SOX17 antibody to extracts made from either human SOX7 or EGFP transfected cells. Furthermore, as shown in Figure 12A-F, the SOX17 antibody exhibits specificity by ICC. The SOX17 antibody clearly labeled the

nuclei of human fibroblast cells transfected with the hSOX17 expression construct (Figure 12A-C) but did not label cells transfected with EGFP alone (Figure 12D-F).

EXAMPLE 4

Validation of SOX17 Antibody as a Marker of Definitive Endoderm

[0171] As evidence that the SOX17 antibody is specific for human SOX17 protein and furthermore marks definitive endoderm, we co-labeled partially differentiated hESCs with SOX17 and AFP antibodies. It has been demonstrated that SOX17 along with AFP and SOX7, a closely related member of the Sox gene family subgroup F (Figure 13), are expressed in visceral endoderm. However, AFP and SOX7 are not expressed in definitive endoderm cells at levels detectable by ICC and can therefore be employed as negative markers for bonifide definitive endoderm cells. As shown in Figure 14A-B, SOX17 antibody labels populations of cells that exist as discrete groupings of cells or are intermingled with AFP positive cells. In particular, Figure 14A shows that small numbers of SOX17 cells were co-labeled with AFP, which could be predicted from the results of *in vivo* studies (Kanai-Azuma et al.) where a subset of visceral endoderm cells was shown to express Sox17 mRNA. However, regions were also found where there were little or no AFP^+ cells in the field of $SOX17^+$ cells (Figure 14B). Similarly, since parietal endoderm has also been reported to express Sox17, antibody co-labeling with Sox17 together with the parietal markers Sparc and/or Thrombomodulin (TM) (Imada et al., 1987) can be used to identify the $SOX17^+$ cells which are parietal endoderm. As shown in Figure 15A-C, Thrombomodulin and SOX17 co-label parietal endoderm cells produced by random differentiation of hES cells. Therefore we would define definitive endoderm with a marker profile $SOX17^{hi}/AFP^{lo}/[TM^{lo}$ or $SPARC^{lo}]$. Accordingly, those cells positive for SOX17 but negative for AFP and negative for TM or SPARC are definitive endoderm.

[0172] As a further proof of the specificity of the $SOX17^{hi}/AFP^{lo}/TM^{lo}/SPARC^{lo}$ marker profile as predictive of definitive endoderm, we quantitatively compared SOX17 and AFP gene expression to the relative number of antibody labeled cells. As shown in Figure 16A, hESCs treated with retinoic acid (visceral endoderm inducer), or Activin A (definitive endoderm inducer), resulted in a 10-fold difference in the level of SOX17 mRNA expression.

This exactly mirrored the 10-fold difference in SOX17 antibody-labeled cell number (Figure 16B). Furthermore, as shown in Figure 17A, Activin A treatment of hESCs suppressed AFP gene expression by 6.8-fold in comparison to no treatment. This was visually reflected by a dramatic decrease in the number of AFP labeled cells in these cultures as shown in Figure 17B. To quantify this further we demonstrated that this approximately 7-fold decrease in AFP gene expression was the result of a similar 7-fold decrease in AFP antibody-labeled cell number as measured by flow cytometry (Figure 18).

[0173] This result is extremely significant in that it indicates that quantitative changes in gene expression as seen by Q-PCR mirror changes in cell type specification as observed by antibody staining, at least in the lineages that were examined here. Incubation of hESCs in the presence of Nodal family members (Nodal, Activin A and Activin B - NAA) resulted in a significant increase in SOX17 antibody-labeled cells over time. By 5 days of continuous activin treatment greater than 50% of the cells were labeled (Figure 19A-F). There were few or no cells labeled with AFP after 5 days of activin treatment.

[0174] In summary, the antibody produced against the carboxy-terminal 242 amino acids of the human SOX17 protein identifies human SOX17 protein on Western blots but does not recognize SOX7, it's closest Sox family relative. The SOX17 antibody recognizes a subset of cells in differentiating hESC cultures that are primarily $SOX17^{+}/AFP^{lo/-}$ (greater than 95% of labeled cells) as well as a small percentage (< 5%) of cells that co-label for SOX17 and AFP (visceral endoderm). Treatment of hESC cultures with activins results in a marked elevation of SOX17 gene expression as well as SOX17 labeled cells and dramatically suppresses the expression of AFP mRNA and the number of cells labeled with AFP antibody.

EXAMPLE 5

Q-PCR Gene Expression Assay

[0175] Real-time quantitative RT-PCR (Q-PCR) was the primary assay used here for screening the effects of various treatments on hESC differentiation. Real-time measurements of gene expression were analyzed for multiple marker genes at multiple time points by Q-PCR. Marker genes characteristic of the desired as well as undesired cell types

were evaluated to gain a better understanding of the overall dynamics of the cellular populations. The strength of Q-PCR analysis includes its extreme sensitivity and relative ease of developing the necessary markers, as the genome sequence is readily available. Furthermore, the extremely high sensitivity of Q-PCR permits detection of gene expression from a relatively small number of cells within a much larger population. In addition, the ability to detect very low levels of gene expression may provide indications for "differentiation bias" within the population. The bias towards a particular differentiation pathway, prior to the overt differentiation of those cellular phenotypes, would likely be unrecognizable using immunocytochemical techniques. For this reason, Q-PCR provides a method of analysis that is complementary to immunocytochemical techniques for screening the success of differentiation treatments. This tool provides a means of evaluating our differentiation protocol successes in a quantitative format at semi-high throughput scales of analysis.

[0176] Our general approach was to perform relative quantitation using SYBR Green chemistry on the Rotor Gene 3000 instrument (Corbett Research) and a two-step RT-PCR format. In this way, we have the option to return to the cDNA samples for analysis of additional marker genes in the future, thus avoiding variability in the reverse transcription efficiency between samples. Primers were designed to lie over exon-exon boundaries or span introns of at least 800 bp when possible, as this has been empirically determined to eliminate amplification from contaminating genomic DNA. When marker genes were employed that do not contain introns or they possess pseudogenes, DNase I treatment of RNA samples was performed.

[0177] We routinely used Q-PCR to measure the gene expression of multiple markers of target and non-target cell types in order to provide a broad profile description of gene expression in the samples. The markers relevant for the early phases of hESC differentiation (specifically ectoderm, mesoderm, definitive endoderm and extra-embryonic endoderm) and for which we have validated primer sets are provided below in Table 1. The human specificity of these primer sets has also been demonstrated. This is an important fact since the hESCs were often grown on mouse feeder layers. Most typically, triplicate samples

were taken for each condition and independently analyzed in duplicate to assess the biological variability associated with each quantitative determination.

[0178] Total RNA was isolated using RNeasy (Qiagen) and quantitated using RiboGreen (Molecular Probes). Reverse transcription from 350-500 ng of total RNA was carried out using the iScript reverse transcriptase kit (BioRad), which contains a mix of oligo-dT and random primers. Each **20 uL** reaction was subsequently diluted up to 100 uL total volume and 3 uL was used in each 10 uL Q-PCR reaction containing 400 nM forward and reverse primers and 5 uL 2X SYBR Green master mix (Qiagen). Two step cycling parameters were used employing a 5 second denature at 85-94°C (specifically selected according to the melting temp of the amplicon for each primer set) followed by a 45 second anneal/extend at 60°C. Fluorescence data was collected during the last 15 seconds of each extension phase. A three point, 10-fold dilution series was used to generate the standard curve for each run and cycle thresholds (Ct's) were converted to quantitative values based on this standard curve (Figure 19). The quantitated values for each sample were normalized to housekeeping gene performance and then average and standard deviations were calculated for triplicate samples. At the conclusion of PCR cycling, a melt curve analysis was performed to ascertain the specificity of the reaction. A single specific product was indicated by a single peak at the T_m appropriate for that PCR amplicon (Figure 20). In addition, reactions performed without reverse transcriptase served as the negative control and do not amplify.

[0179] A first step in establishing the Q-PCR methodology was validation of appropriate housekeeping genes (HGs) in the experimental system. Since the HG was used to normalize across samples for the RNA input, RNA integrity and RT efficiency, it was of value that the HG exhibited a constant level of expression over time in all sample types in order for the normalization to be meaningful. We measured the expression levels of *Cyclophilin G, hypoxanthine phosphoribosyltransferase 1 (HPRT), beta-2-microglobulin, hydroxymethylbiane synthase (HMBS), TATA-binding protein (TBP), and glucoronidase beta (GUS)* in differentiating hESCs. Our results indicated that *beta-2-microglobulin* expression levels increased over the course of differentiation and therefore we excluded the use of this gene for normalization. The other genes exhibited consistent expression levels over time as well as across treatments. We routinely used both Cyclophilin G and GUS to calculate a

normalization factor for all samples. The use of multiple HGs simultaneously reduces the variability inherent to the normalization process and increases the reliability of the relative gene expression values (Vandesompele et al., 2002).

[0180] We have utilized Q-PCR to determine the relative gene expression levels of many marker genes across samples receiving different experimental treatments. The marker genes employed have been chosen because they exhibit enrichment in specific populations representative of the early germ layers and in particular have focused on sets of genes that are differentially expressed in definitive endoderm and extra-embryonic endoderm. These genes as well as their relative enrichment profiles are highlighted in Table 1.

TABLE 1

Germ Layer	Gene	Expression Domains	References
Endoderm	SOX17	definitive, visceral and parietal endoderm	
	MIXL1	endoderm and mesoderm	
	GATA4	definitive and primitive endoderm	
	HNF3b	definitive endoderm and primitive endoderm, mesoderm, neural plate	
Extra-embryonic	SOX7	visceral endoderm	
	AFP	visceral endoderm, liver	
	SPARC	parietal endoderm	
	TM	parietal endoderm/trophectoderm	
Ectoderm	ZIC1	neural tube, neural progenitors	
Mesoderm	BRACH	nascent mesoderm	

[0181] Since many genes are expressed in more than one germ layer it is important to quantitatively compare expression levels of many genes within the same experiment. SOX17 is expressed in definitive endoderm and to a smaller extent in visceral and parietal endoderm (Kanai-Azuma et al., 2002), SOX7 and AFP are expressed in visceral endoderm at this early developmental time point (Katoh, 2002; Shiozawa et al., 1996; Takash et al., 2001; Taniguchi et al., 1999), SPARC (Futaki et al., 2003; Harris and Childs, 2002; Howe et al., 1988), and TM (Weiler-Guettler et al., 1996; Weiler-Guettler et al., 1992) are expressed in parietal endoderm and Brachyury (Beddington et al., 1992; Smith, 1997; Smith et al., 1997; Technau, 2001; Willison, 1990) is expressed in early mesoderm.

[0182] Definitive endoderm cells were predicted to express high levels of SOX17 mRNA and low levels of AFP and SOX7 (visceral endoderm), SPARC (parietal endoderm) and Brachyury (mesoderm). In addition, ZIC1 (Ogura et al., 2001; Rohr et al., 1999) was used here to further rule out induction of early ectoderm. Finally, GATA4 and HNF3b were expressed in both definitive and extra-embryonic endoderm, and thus, correlate with SOX17 expression in definitive endoderm (Table 1). A representative experiment is shown in Figures 21-24 which demonstrates how the marker genes described in Table 1 correlate with each other among the various samples, thus highlighting specific patterns of differentiation to

definitive endoderm and extra-embryonic endoderm as well as to mesodermal and neural cell types.

[0183] From this data it is clear that increasing doses of activin results in increasing SOX17 gene expression. Further this SOX17 expression appears to predominantly represent definitive endoderm as opposed to extra-embryonic endoderm. This conclusion stems from the observation that SOX17 gene expression is inversely correlated with AFP, SOX7, and SPARC gene expression.

EXAMPLE 6

Directed Differentiation of Human ES Cells to Definitive Endoderm

[0184] Human ES cell cultures will randomly differentiate if they are cultured under conditions that do not actively maintain their undifferentiated state. This heterogeneous differentiation results in production of extra-embryonic endoderm cells composed of both parietal and visceral endoderm (AFP, SPARC and SOX7 expression) as well as early ectodermal and mesodermal derivatives as marked by ZIC1 and Nestin (ectoderm) and Brachyury (mesoderm) expression. Definitive endoderm cell appearance has not traditionally been examined or specified for lack of specific antibody markers in ES cell cultures. As such and by default, early definitive endoderm production in ES cell cultures has not been well studied. Since no good antibody reagents for definitive endoderm cells have been available, most of the characterization has focused on ectoderm and extra-embryonic endoderm. Overall, there are significantly greater numbers of extra-embryonic and neurectodermal cell types in comparison to $SOX17^{hi}$ definitive endoderm cells in randomly differentiated ES cell cultures.

[0185] As undifferentiated hESC colonies expand on a bed of fibroblast feeders the edges of the colony take on alternative morphologies that are distinct from those cells residing within the interior of the colony. Many of these outer edge cells can be distinguished by their less uniform, larger cell body morphology and by the expression of higher levels of OCT4 (Figure 25A-C). It has been described that as ES cells begin to differentiate they alter the levels of OCT4 expression up or down relative to undifferentiated ES cells (Niwa, 2001).

Alteration of OCT4 levels above or below the undifferentiated threshold may signify the initial stages of differentiation away from the pluripotent state (Niwa, 2001).

[0186] When undifferentiated colonies were examined by SOX17 immunocytochemistry, occasionally small 10-15-cell clusters of SOX17-positive cells were detected at random locations on the periphery and at the junctions between undifferentiated ESC colonies (Figure 26A&D). As noted above, these scattered pockets of outer colony edges appeared to be some of the first cells to differentiate away from the classical ESC morphology as the colony expanded in size and became more crowded. Younger, smaller fully undifferentiated colonies (< 1mm; 4-5 days old) showed no SOX17 positive cells within or at the edges of the colonies while older, larger colonies (1-2 mm diameter, > 5days old) had sporadic isolated patches of SOX17 positive, AFP negative cells at the periphery of some colonies or in regions interior to the edge (Figure 26) that were differentiated away from classical hESC morphology shown previously (Figure 2). Given that this was the first development of an effective SOX17 antibody, definitive endoderm cells generated in such early "undifferentiated" ESC cultures have never been previously demonstrated.

[0187] Based on negative correlations of SOX17 and SPARC gene expression levels by Q-PCR, the vast majority of these SOX17 positive, AFP negative cells will be negative for parietal markers by antibody co-labeling. This was specifically demonstrated for TM expressing parietal endoderm cells as shown in Figure 27A-B. Exposure to Nodal factors Activin A and B resulted in a dramatic decrease in the intensity to TM expression and the number of TM positive cells. By triple labeling using Sox17, AFP and TM antibodies on an activin treated culture, many clusters of Sox17 positive cells which are also negative for AFP and TM were observed (Figure 37). These are the first cellular demonstrations of SOX17 positive definitive endoderm cells in differentiating ESC cultures (Figures 37 & 28).

[0188] With the SOX17 antibody and Q-PCR tools described above we have explored a number of procedures capable of efficiently programming ESCs to become $SOX17^{hi}/AFP^{lo}$ / $SPARC/TM^{lo}$ definitive endoderm cells (Figure 28). We applied a variety of differentiation protocols aimed at increasing the number and proliferative capacity of these cells as measured at the population level by Q-PCR for SOX17 gene expression and at the level of individual cells by antibody labeling of SOX17 protein.

[0189] As described earlier, the Nodal/activin subgroup of the TGFβ superfamily of factors (Figure 29) (Chang et al., 2002; Miyazono et al., 2001; Zhao, 2003) has been investigated with regard to mesoderm and endoderm induction in vivo. It is now generally believed that the Nodal-related TGFβ family member signaling molecules and their receptors are responsible for the induction of mesendoderm i.e. mesoderm and definitive endoderm during gastrulation (Conlon et al., 1994; Feldman et al., 1998; Hogan, 1996; Tremblay et al., 2000; Varlet et al., 1997; Zhou et al., 1993). However, little data has been generated analyzing Nodal/activin/BMP effects in vitro with regard to identification of definitive endoderm cells as created from embryonic stem cells.

[0190] As shown in Figure 30, addition of Activin A at 100ng/ml resulted in a 19-fold induction of SOX17 gene expression vs. undifferentiated hESCs by day 4 of differentiation. Adding Activin B, a second member of the activin family, together with Activin A, resulted in a 37-fold induction over undifferentiated hESCs by day 4 of combined activin treatment. Finally, adding a third member of the TGFβ family from the Nodal/Activin and BMP subgroups, BMP4, together with Activin A and Activin B, increased the fold induction to 57 times that of undifferentiated hESCs (Figure 30). When SOX17 induction with activins and BMP was compared to no factor medium controls 5-, 10-, and 15-fold inductions resulted at the 4-day time point. By five days of triple treatment with Activins A, B and BMP, SOX17 was induced more than 70 times higher than hESCs. These data indicate that higher doses and longer treatment times of the Nodal/activin TGFβ family members results in increased expression of SOX17.

[0191] *In vivo*, mesendoderm gives rise to endoderm and mesoderm. The relative proportions of endoderm and mesoderm are dependent on the level of Nodal signaling as controlled by dose and length of exposure. Higher levels of nodal signaling result in more endoderm at the expense of mesoderm and vice-versa for lower Nodal signaling. (Feldman et al., 1998; Kikuchi et al., 2001; Schier, 2003; Vincent et al., 2003). However, no data exists showing that the combination of Nodal and related molecules Activin A, B and BMP facilitates the expression of SOX17 and definitive endoderm formation *in vivo* or *in vitro*. Furthermore, addition of BMP results in an improved SOX17 induction possibly through the further induction of Cripto, the Nodal co-receptor (Beck et al., 2002; Kumar et al., 2001).

Again, the combination of Activins A and B together with BMP4 have not previously been shown to result in additive increases in SOX17 induction and hence definitive endoderm formation. BMP4 addition for prolonged periods (>4 days), in combination with Activin A and B may induce SOX17 in parietal and visceral endoderm as well as definitive endoderm (through Cripto receptor induction). It may therefore be valuable to remove BMP4 from the treatment within 4 days of addition.

[0192] To determine the effect of TGFβ factor treatment at the individual cell level, a time course of TGFβ factor addition was examined using SOX17 antibody labeling. As previously shown in Figure 19A-F, there was a dramatic increase in the relative number of SOX17 labeled cells over time. The relative quantification (Figure 31) shows more than a 20-fold increase in SOX17-labeled cells. This result indicates that both the numbers of cells as well SOX17 gene expression level are increasing with time of TGFβ factor exposure. As shown in Figure 32, after four days of exposure to Nodal, Activin A, Activin B and BMP4, the level of SOX17 induction reached 168-fold over undifferentiated hESCs. Figure 33 shows that the relative number of SOX17-positive cells was also dose responsive. Activin A doses of 100 ng/mL or more were capable of potently inducing SOX17 gene expression and cell number.

[0193] In addition to the TGFβ family members, there has also been some indication that the Wnt family of molecules may play a role in specification and/or maintenance of definitive endoderm (Lickert et al., 2002; Rodaway and Patient, 2001; Rodaway et al., 1999). The use of Wnt molecules was also beneficial for the differentiation of hESCs to definitive endoderm as indicted by the increased SOX17 gene expression in samples that were treated with activins plus Wnt3a over that of activins alone (Figure 34).

[0194] All of the experiments described above were performed using tissue culture medium containing 10% serum with added factors. Interestingly, the concentration of serum had an effect on the level of SOX17 expression in the presence of added activins as shown in Figure 35A-C. When serum levels were reduced from 10% to 2%, SOX17 expression tripled in the presence of Activins A and B.

[0195] Finally, we demonstrated that activin induced $SOX17^{+}$ cells divide in culture as depicted in Figure 36A-D. The arrows show cells labeled with

SOX17/PCNA/DAPI that are in mitosis as evidenced by the PCNA/DAPI-labeled mitotic plate pattern and the phase contrast mitotic profile.

[0196] The methods, compositions, and devices described herein are presently representative of preferred embodiments and are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the disclosure. Accordingly, it will be apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

[0197] As used in the claims below and throughout this disclosure, by the phrase "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

References

[0198] Numerous literature and patent references have been cited in the present application. All references cited are incorporated by reference herein in their entireties.

[0199] For some references, the complete citation is in the body of the text. For other references the citation in the body of the text is by author and year, the complete citation being as follows:

[0200] Alexander, J., Rothenberg, M., Henry, G. L., and Stainier, D. Y. (1999). casanova plays an early and essential role in endoderm formation in zebrafish. Dev Biol *215*, 343-357.

[0201] Alexander, J., and Stainier, D. Y. (1999). A molecular pathway leading to endoderm formation in zebrafish. Curr Biol *9*, 1147-1157.

[0202] Aoki, T. O., Mathieu, J., Saint-Etienne, L., Rebagliati, M. R., Peyrieras, N., and Rosa, F. M. (2002). Regulation of nodal signalling and mesendoderm formation by TARAM-A, a TGFbeta-related type I receptor. Dev Biol *241*, 273-288.

[0203] Beck, S., Le Good, J. A., Guzman, M., Ben Haim, N., Roy, K., Beermann, F., and Constam, D. B. (2002). Extra-embryonic proteases regulate Nodal signalling during gastrulation. Nat Cell Biol *4*, 981-985.

[0204] Beddington, R. S., Rashbass, P., and Wilson, V. (1992). Brachyury--a gene affecting mouse gastrulation and early organogenesis. Dev Suppl, 157-165.

[0205] Bongso, A., Fong, C. Y., Ng, S. C., and Ratnam, S. (1994). Isolation and culture of inner cell mass cells from human blastocysts. Hum Reprod *9*, 2110-2117.

[0206] Chang, H., Brown, C. W., and Matzuk, M. M. (2002). Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev *23*, 787-823.

[0207] Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Herrmann, B., and Robertson, E. J. (1994). A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development *120*, 1919-1928.

[0208] Dougan, S. T., Warga, R. M., Kane, D. A., Schier, A. F., and Talbot, W. S. (2003). The role of the zebrafish nodal-related genes squint and cyclops in patterning of mesendoderm. Development *130*, 1837-1851.

[0209] Feldman, B., Gates, M. A., Egan, E. S., Dougan, S. T., Rennebeck, G., Sirotkin, H. I., Schier, A. F., and Talbot, W. S. (1998). Zebrafish organizer development and germ-layer formation require nodal-related signals. Nature *395*, 181-185.

[0210] Futaki, S., Hayashi, Y., Yamashita, M., Yagi, K., Bono, H., Hayashizaki, Y., Okazaki, Y., and Sekiguchi, K. (2003). Molecular basis of constitutive production of basement membrane components: Gene expression profiles of engelbreth-holm-swarm tumor and F9 embryonal carcinoma cells. J Biol Chem.

[0211] Grapin-Botton, A., and Melton, D. A. (2000). Endoderm development: from patterning to organogenesis. Trends Genet *16*, 124-130.

[0212] Harris, T. M., and Childs, G. (2002). Global gene expression patterns during differentiation of F9 embryonal carcinoma cells into parietal endoderm. Funct Integr Genomics *2*, 105-119.

[0213] Hogan, B. L. (1996). Bone morphogenetic proteins in development. Curr Opin Genet Dev *6*, 432-438.

[0214] Hogan, B. L. (1997). Pluripotent embryonic cells and methods of making same (U.S.A., Vanderbilt University).

[0215] Howe, C. C., Overton, G. C., Sawicki, J., Solter, D., Stein, P., and Strickland, S. (1988). Expression of SPARC/osteonectin transcript in murine embryos and gonads. Differentiation *37*, 20-25.

[0216] Hudson, C., Clements, D., Friday, R. V., Stott, D., and Woodland, H. R. (1997). Xsox17alpha and -beta mediate endoderm formation in Xenopus. Cell *91*, 397-405.

[0217] Imada, M., Imada, S., Iwasaki, H., Kume, A., Yamaguchi, H., and Moore, E. E. (1987). Fetomodulin: marker surface protein of fetal development which is modulatable by cyclic AMP. Dev Biol *122*, 483-491.

[0218] Kanai-Azuma, M., Kanai, Y., Gad, J. M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., Yonekawa, H., Yazaki, K., Tam, P. P., and Hayashi, Y. (2002). Depletion of definitive gut endoderm in Sox17-null mutant mice. Development *129*, 2367-2379.

[0219] Katoh, M. (2002). Expression of human SOX7 in normal tissues and tumors. Int J Mol Med *9*, 363-368.

[0220] Kikuchi, Y., Agathon, A., Alexander, J., Thisse, C., Waldron, S., Yelon, D., Thisse, B., and Stainier, D. Y. (2001). casanova encodes a novel Sox-related protein necessary and sufficient for early endoderm formation in zebrafish. Genes Dev *15*, 1493-1505.

[0221] Kimelman, D., and Griffin, K. J. (2000). Vertebrate mesendoderm induction and patterning. Curr Opin Genet Dev *10*, 350-356.

[0222] Kumar, A., Novoselov, V., Celeste, A. J., Wolfman, N. M., ten Dijke, P., and Kuehn, M. R. (2001). Nodal signaling uses activin and transforming growth factor-beta receptor-regulated Smads. J Biol Chem *276*, 656-661.

[0223] Labosky, P. A., Barlow, D. P., and Hogan, B. L. (1994a). Embryonic germ cell lines and their derivation from mouse primordial germ cells. Ciba Found Symp *182*, 157-168; discussion 168-178.

[0224] Labosky, P. A., Barlow, D. P., and Hogan, B. L. (1994b). Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. Development *120*, 3197-3204.

[0225] Lickert, H., Kutsch, S., Kanzler, B., Tamai, Y., Taketo, M. M., and Kemler, R. (2002). Formation of multiple hearts in mice following deletion of beta-catenin in the embryonic endoderm. Dev Cell *3*, 171-181.

[0226] Lu, C. C., Brennan, J., and Robertson, E. J. (2001). From fertilization to gastrulation: axis formation in the mouse embryo. Curr Opin Genet Dev *11*, 384-392.

[0227] Miyazono, K., Kusanagi, K., and Inoue, H. (2001). Divergence and convergence of TGF-beta/BMP signaling. J Cell Physiol *187*, 265-276.

[0228] Niwa, H. (2001). Molecular mechanism to maintain stem cell renewal of ES cells. Cell Struct Funct *26*, 137-148.

[0229] Ogura, H., Aruga, J., and Mikoshiba, K. (2001). Behavioral abnormalities of Zic1 and Zic2 mutant mice: implications as models for human neurological disorders. Behav Genet *31*, 317-324.

[0230] Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol *18*, 399-404.

[0231] Rodaway, A., and Patient, R. (2001). Mesendoderm. an ancient germ layer? Cell *105*, 169-172.

[0232] Rodaway, A., Takeda, H., Koshida, S., Broadbent, J., Price, B., Smith, J. C., Patient, R., and Holder, N. (1999). Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-beta family signals and discrimination of mesoderm and endoderm by FGF. Development *126*, 3067-3078.

[0233] Rohr, K. B., Schulte-Merker, S., and Tautz, D. (1999). Zebrafish zic1 expression in brain and somites is affected by BMP and hedgehog signalling. Mech Dev *85*, 147-159.

[0234] Schier, A. F. (2003). Nodal signaling in vertebrate development. Annu Rev Cell Dev Biol *19*, 589-621.

[0235] Schoenwolf, G. C., and Smith, J. L. (2000). Gastrulation and early mesodermal patterning in vertebrates. Methods Mol Biol *135*, 113-125.

[0236] Shamblott, M. J., Axelman, J., Wang, S., Bugg, E. M., Littlefield, J. W., Donovan, P. J., Blumenthal, P. D., Huggins, G. R., and Gearhart, J. D. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci U S A *95*, 13726-13731.

[0237] Shapiro, A. M., Lakey, J. R., Ryan, E. A., Korbutt, G. S., Toth, E., Warnock, G. L., Kneteman, N. M., and Rajotte, R. V. (2000). Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med *343*, 230-238.

[0238] Shapiro, A. M., Ryan, E. A., and Lakey, J. R. (2001a). Pancreatic islet transplantation in the treatment of diabetes mellitus. Best Pract Res Clin Endocrinol Metab *15*, 241-264.

[0239] Shapiro, J., Ryan, E., Warnock, G. L., Kneteman, N. M., Lakey, J., Korbutt, G. S., and Rajotte, R. V. (2001b). Could fewer islet cells be transplanted in type 1 diabetes? Insulin independence should be dominant force in islet transplantation. Bmj *322*, 861.

[0240] Shiozawa, M., Hiraoka, Y., Komatsu, N., Ogawa, M., Sakai, Y., and Aiso, S. (1996). Cloning and characterization of Xenopus laevis xSox7 cDNA. Biochim Biophys Acta *1309*, 73-76.

[0241] Smith, J. (1997). Brachyury and the T-box genes. Curr Opin Genet Dev *7*, 474-480.

[0242] Smith, J. C., Armes, N. A., Conlon, F. L., Tada, M., Umbhauer, M., and Weston, K. M. (1997). Upstream and downstream from Brachyury, a gene required for vertebrate mesoderm formation. Cold Spring Harb Symp Quant Biol *62*, 337-346.

[0243] Takash, W., Canizares, J., Bonneaud, N., Poulat, F., Mattei, M. G., Jay, P., and Berta, P. (2001). SOX7 transcription factor: sequence, chromosomal localisation, expression, transactivation and interference with Wnt signalling. Nucleic Acids Res *29*, 4274-4283.

[0244] Taniguchi, K., Hiraoka, Y., Ogawa, M., Sakai, Y., Kido, S., and Aiso, S. (1999). Isolation and characterization of a mouse SRY-related cDNA, mSox7. Biochim Biophys Acta *1445*, 225-231.

[0245] Technau, U. (2001). Brachyury, the blastopore and the evolution of the mesoderm. Bioessays *23*, 788-794.

[0246] Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.

[0247] Tremblay, K. D., Hoodless, P. A., Bikoff, E. K., and Robertson, E. J. (2000). Formation of the definitive endoderm in mouse is a Smad2-dependent process. Development *127*, 3079-3090.

[0248] Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol *3*, RESEARCH0034.

[0249] Varlet, I., Collignon, J., and Robertson, E. J. (1997). nodal expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. Development *124*, 1033-1044.

[0250] Vincent, S. D., Dunn, N. R., Hayashi, S., Norris, D. P., and Robertson, E. J. (2003). Cell fate decisions within the mouse organizer are governed by graded Nodal signals. Genes Dev *17*, 1646-1662.

[0251] Weiler-Guettler, H., Aird, W. C., Rayburn, H., Husain, M., and Rosenberg, R. D. (1996). Developmentally regulated gene expression of thrombomodulin in postimplantation mouse embryos. Development *122*, 2271-2281.

[0252] Weiler-Guettler, H., Yu, K., Soff, G., Gudas, L. J., and Rosenberg, R. D. (1992). Thrombomodulin gene regulation by cAMP and retinoic acid in F9 embryonal carcinoma cells. Proceedings Of The National Academy Of Sciences Of The United States Of America *89*, 2155-2159.

[0253] Wells, J. M., and Melton, D. A. (1999). Vertebrate endoderm development. Annu Rev Cell Dev Biol *15*, 393-410.

[0254] Wells, J. M., and Melton, D. A. (2000). Early mouse endoderm is patterned by soluble factors from adjacent germ layers. Development *127*, 1563-1572.

[0255] Willison, K. (1990). The mouse Brachyury gene and mesoderm formation. Trends Genet *6*, 104-105.

[0256] Zhao, G. Q. (2003). Consequences of knocking out BMP signaling in the mouse. Genesis *35*, 43-56.

[0257] Zhou, X., Sasaki, H., Lowe, L., Hogan, B. L., and Kuehn, M. R. (1993). Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. Nature *361*, 543-547.

WHAT IS CLAIMED IS:

1. A composition comprising stem cells and definitive endoderm cells, wherein at least 5 definitive endoderm cells are present for about every 95 stem cells present in said composition.

2. The composition of Claim 1, wherein at least 10 definitive endoderm cells are present for about every 90 stem cells present in said composition.

3. The composition of Claim 1, wherein at least 20 definitive endoderm cells are present for about every 80 stem cells present in said composition.

4. The composition of Claim 1, wherein at least 30 definitive endoderm cells are present for about every 70 stem cells present in said composition.

5. The composition of Claim 1, wherein at least 40 definitive endoderm cells are present for about every 60 stem cells present in said composition.

6. The composition of Claim 1, wherein at least 50 definitive endoderm cells are present for about every 50 stem cells present in said composition.

7. The composition of Claim 1, wherein at least 60 definitive endoderm cells are present for about every 40 stem cells present in said composition.

8. The composition of Claim 1, wherein at least 70 definitive endoderm cells are present for about every 30 stem cells present in said composition.

9. The composition of Claim 1, wherein at least 80 definitive endoderm cells are present for about every 20 stem cells present in said composition.

10. The composition of Claim 1, wherein at least 90 definitive endoderm cells are present for about every 10 stem cells present in said composition.

11. The composition of Claim 1, wherein at least 95 definitive endoderm cells are present for about every 5 stem cells present in said composition.

12. The composition of Claim 1, wherein said stem cells are embryonic stem cells.

13. The composition of Claim 12, wherein said embryonic stem cells are derived from the inner cell mass of an embryo.

14. The composition of Claim 12, wherein said embryonic stem cells are derived from the gonadal ridges of an embryo.

15. The composition of Claim 1, wherein said stem cells are of human origin.

16. The composition of Claim 1, wherein said definitive endoderm cells are of human origin.

17. The composition of Claim 1, wherein said definitive endoderm cells express the SOX17 gene.

18. The composition of Claim 17, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the SOX7 gene.

19. The composition of Claim 18, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the AFP gene.

20. The composition of Claim 19, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the SPARC gene.

21. The composition of Claim 20, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the Thrombomodulin gene.

22. The composition of Claim 21, wherein said definitive endoderm cells express the MIXL1 gene.

23. The composition of Claim 21, wherein said definitive endoderm cells express the GATA4 gene.

24. The composition of Claim 21, wherein said definitive endoderm cells express the HNF3b gene.

25. The composition of Claim 1 further comprising a growth factor of the Nodal/Activin subgroup of the TGFβ superfamily.

26. The composition of Claim 1 further comprising a growth factor of the BMP subgroup of the TGFβ superfamily.

27. The composition of Claim 1 further comprising a growth factor selected from the group consisting of Nodal, Activin A, Activin B, BMP4, Wnt3a and combinations thereof.

28. The composition of Claim 1 further comprising Nodal, Activin A, Activin B and BMP4.

29. A substantially purified definitive endoderm cell.

30. The definitive endoderm cell of Claim 29, wherein said cell is derived from an embryonic stem cell.

31. The definitive endoderm cell of Claim 29, wherein said cell expresses the SOX17 gene.

32. The definitive endoderm cell of Claim 31, wherein the expression of the SOX17 gene is greater than the expression of the SOX7 gene.

33. The definitive endoderm cell of Claim 32, wherein the expression of the SOX17 gene is greater than the expression of the AFP gene.

34. The definitive endoderm cell of Claim 33, wherein the expression of the SOX17 gene is greater than the expression of the SPARC gene.

35. The definitive endoderm cell of Claim 34, wherein the expression of the SOX17 gene is greater than the expression of the Thrombomodulin gene.

36. The definitive endoderm cell of Claim 35, wherein said cell expresses the MIXL1 gene.

37. The definitive endoderm cell of Claim 35, wherein said cell expresses the GATA4 gene.

38. The definitive endoderm cell of Claim 35, wherein said cell expresses the HNF3b gene.

39. The definitive endoderm cell of Claim 29, wherein said cell is of human origin.

40. A method for producing definitive endoderm cells, said method comprising:

obtaining a stem cell culture; and

providing to said culture a growth factor of the TGFβ superfamily in an amount sufficient to promote differentiation of at least a portion of said stem cell culture to definitive endoderm cells.

41. The method of Claim 40, wherein at least 5 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

42. The method of Claim 40, wherein at least 10 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

43. The method of Claim 40, wherein at least 20 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

44. The method of Claim 40, wherein at least 30 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

45. The method of Claim 40, wherein at least 40 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

46. The method of Claim 40, wherein at least 50 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

47. The method of Claim 40, wherein at least 60 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

48. The method of Claim 40, wherein at least 70 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

49. The method of Claim 40, wherein at least 80 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

50. The method of Claim 40, wherein at least 90 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

51. The method of Claim 40, wherein at least 95 percent of the stem cells in said culture differentiates into said definitive endoderm cells.

52. The method of Claim 40, wherein the stem cells in said culture are embryonic stem cells.

53. The method of Claim 52, wherein said embryonic stem cells are derived from the inner cell mass of an embryo.

54. The method of Claim 52, wherein said embryonic stem cells are derived from the gonadal ridges of an embryo.

55. The method of Claim 40, wherein said stem cells in said culture are of human origin.

56. The method of Claim 40, wherein said definitive endoderm cells are of human origin.

57. The method of Claim 40, wherein said definitive endoderm cells express the SOX17 gene.

58. The method of Claim 57, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the SOX7 gene.

59. The method of Claim 58, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the AFP gene.

60. The method of Claim 59, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the SPARC gene.

61. The method of Claim 60, wherein the expression of the SOX17 gene in said definitive endoderm cells is greater than the expression of the Thrombomodulin gene.

62. The method of Claim 61, wherein said definitive endoderm cells express the MIXL1 gene.

63. The method of Claim 61, wherein said definitive endoderm cells express the GATA4 gene.

64. The method of Claim 61, wherein said definitive endoderm cells express the HNF3b gene.

65. The method of Claim 40, wherein said growth factor is of the Nodal/Activin subgroup of the TGFβ superfamily.

66. The method of Claim 40, wherein said growth factor is of the BMP subgroup of the TGFβ superfamily.

67. The method of Claim 40, wherein said growth factor is selected from the group consisting of Nodal, Activin A, Activin B, BMP4 and combinations thereof.

68. The method of Claim 67 further comprising the growth factor Wnt3a.

69. The method of Claim 40, wherein a plurality of growth factors of the TGFβ superfamily is provided.

70. The method of Claim 69, wherein the plurality of growth factors comprises Nodal, Activin A, Activin B and BMP4.

71. The method of Claim 40, wherein said growth factor is BMP4.

72. The method of Claim 71 further comprising the step of removing BMP4 within approximately 4 days after its addition.

73. The method of Claim 40 wherein said growth factor is provided in a concentration of at least 10 ng/ml.

74. The method of Claim 40, wherein said growth factor is provided in a concentration of at least 25 ng/ml.

75. The method of Claim 40, wherein said growth factor is provided in a concentration of at least 50 ng/ml.

76. The method of Claim 40, wherein said growth factor is provided in a concentration of at least 75 ng/ml.

77. The method of Claim 40, wherein said growth factor is provided in a concentration of at least 100 ng/ml.

78. The method of Claim 40, wherein said growth factor is provided in a concentration of at least 200 ng/ml.

79. The method of Claim 40, wherein said growth factor is provided in a concentration of at least 300 ng/ml.

80. The method of Claim 40, wherein said growth factor is provided in a concentration of at least 400 ng/ml.

81. The method of Claim 40, wherein said growth factor is provided in a concentration of at least 500 ng/ml.

82. The method of Claim 40, wherein said growth factor is provided in a concentration of at least 1000 ng/ml.

83. A definitive endoderm cell produced by the method of Claim 40.

84. An antibody which binds to SOX17.

85. The antibody of Claim 84, wherein said SOX17 is human SOX17.

86. The antibody of Claim 84, wherein said antibody is a polyclonal.

87. The antibody of Claim 84, wherein said antibody is a monoclonal antibody.

88. A method of increasing the expression of the SOX17 gene product in a stem cell comprising contacting said stem cell with a growth factor of the TGFβ superfamily in an amount sufficient to increase expression of the SOX17 gene product.

89. The method of Claim 88, wherein the stem cell is an embryonic stem cell.

90. The method of Claim 89, wherein said embryonic stem cell is derived from the inner cell mass of an embryo.

91. The method of Claim 89, wherein said embryonic stem cell is derived from the gonadal ridges of an embryo.

92. The method of Claim 88, wherein said stem cell is of human origin.

93. The method of Claim 88, wherein said growth factor is selected from the group consisting of Nodal, Activin A, Activin B, BMP4 and combinations thereof.

94. The method of Claim 93 further comprising the growth factor Wnt3a.

95. The method of Claim 88, wherein a plurality of growth factors of the TGFβ superfamily is provided.

96. The method of Claim 95, wherein the plurality of growth factors comprises Nodal, Activin A, Activin B and BMP4.

97. The method of Claim 88, wherein said growth factor is BMP4.

98. The method of Claim 97 further comprising the step of removing BMP4 within approximately 4 days after its addition.

DEFINITIVE ENDODERM

Abstract of the Disclosure

Disclosed herein are compositions of substantially purified definitive endoderm cells and compositions comprising both stem cells and definitive endoderm cells. Also disclosed herein are methods of producing definitive endoderm cells from stem cells and methods of using the definitive endoderm cells produced.

S:\DOCS\JLH\JLH-2445.DOC:122203

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

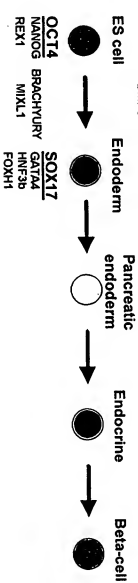

Step-wise β-cell differentiation
TGFβ family
Activins
Nodals
BMPs
ES cell
OCT4
NANOG
REX1
Endoderm
BRACHYURY
MIXL1
SOX17
GATA4
HNF3b
FOXH1
Pancreatic endoderm
Endocrine
Beta-cell

FIGURE 1

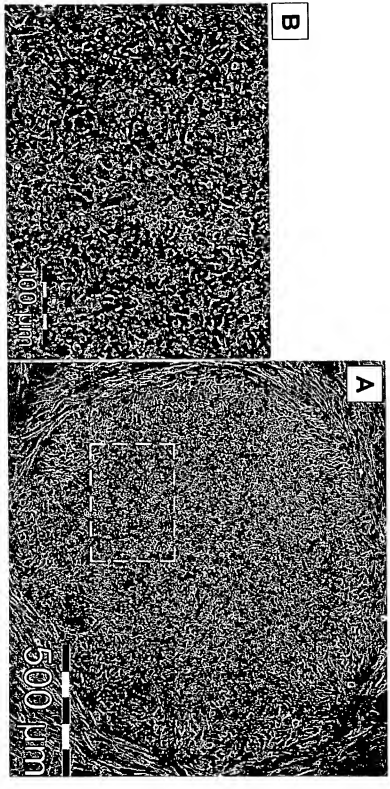
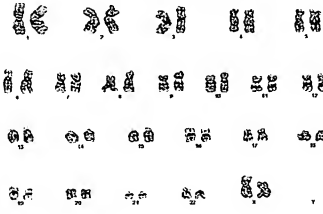


FIGURE 2

hESCyT-25 Chromosome Analysis at Passage 10



hESCyT-25 Chromosome Analysis at Passage 28

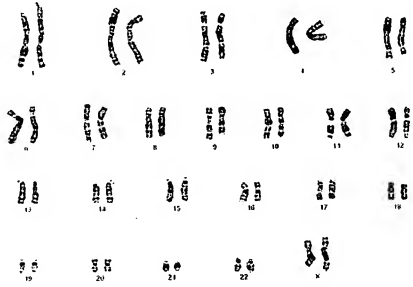


FIGURE 3

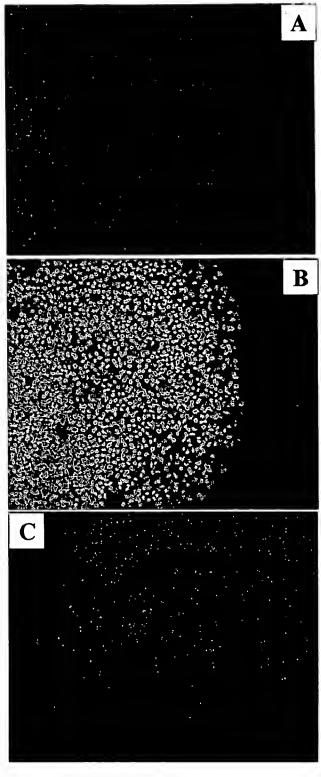


FIGURE 4

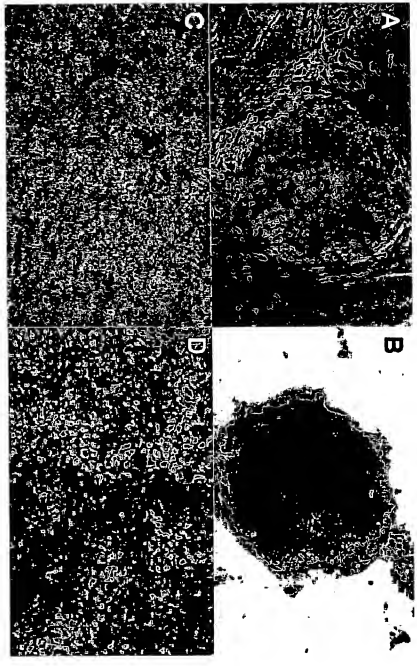


FIGURE 5

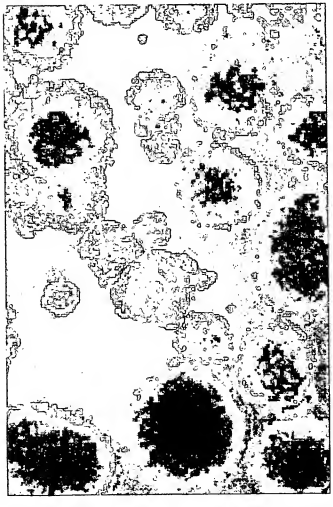
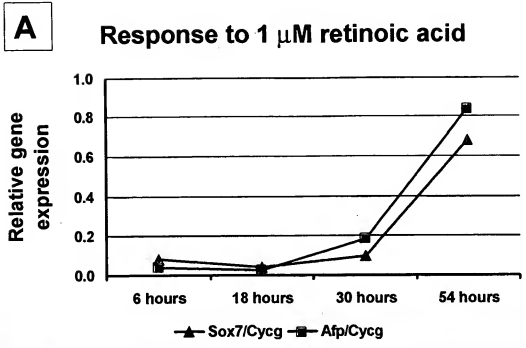


FIGURE 6

FIGURE 7


A
Response to 1 μM retinoic acid
Relative gene expression
1.0
0.8
0.6
0.4
0.2
0.0
6 hours
18 hours
30 hours
54 hours
Sox7/Cycg
Afp/Cycg

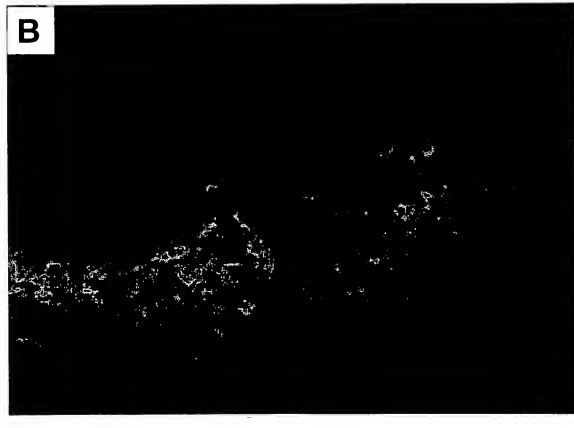

B

FIGURE 8

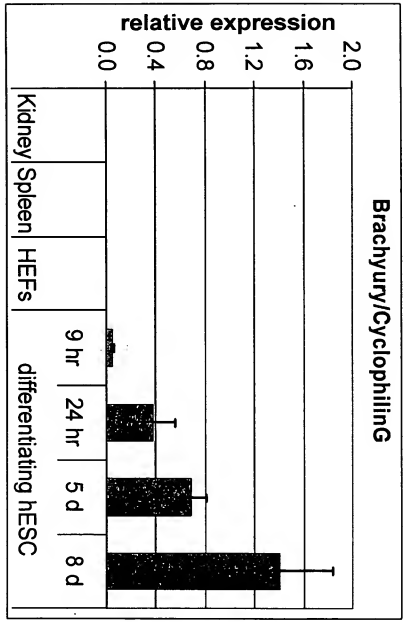

Brachyury/CyclophilinG
relative expression
2.0
1.6
1.2
0.8
0.4
0.0
Kidney
Spleen
HEFs
9 hr
24 hr
5 d
8 d
differentiating hESC

FIGURE 9

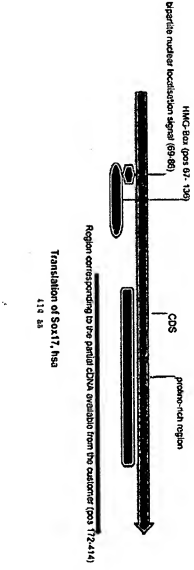

bipartite nuclear localisation signal (69-85)
HMG-Box (pos 97- 139)
CDS
proline-rich region
Region corresponding to the partial cDNA available from the customer (pos 172-414)
Translation of Sox17.hsa
414 aa

FIGURE 10

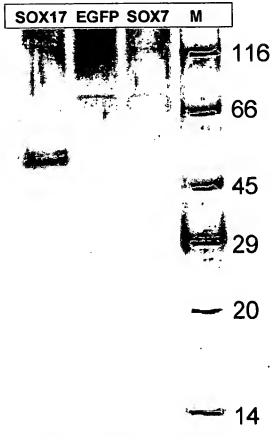


FIGURE 11

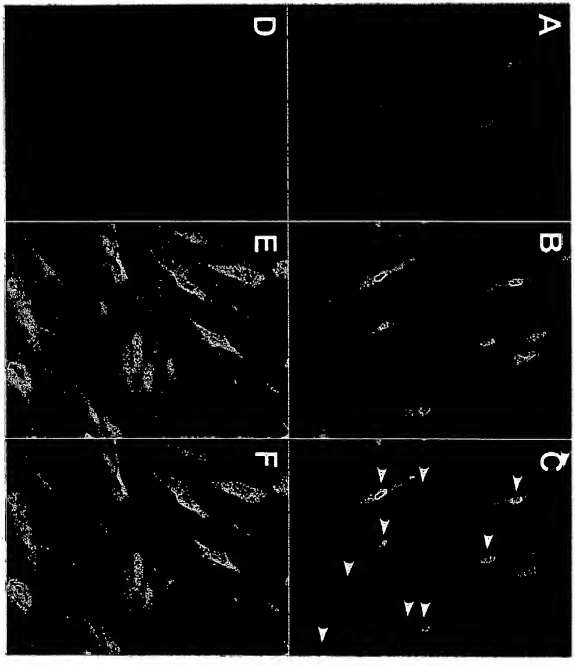


FIGURE 12


dr-SOXF
mo-SOX18
mo-SOX7
xe-SOX7
xe-SOX17B
xe-SOX17A
mo-SOX17
dr-SOXE
mf/h-SOX10
ch-SOX9
fr-SOX8
mf/h-SOX8
fr-SOX9
mf/h-SOX9
af-SOX9
GroupE
GroupF

FIGURE 13

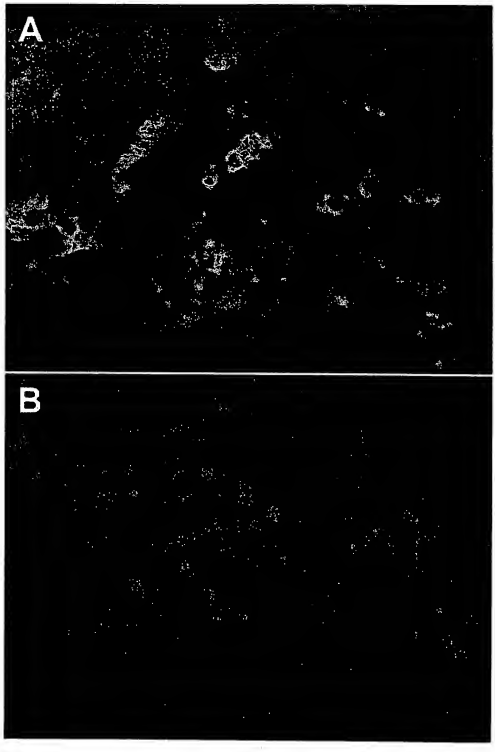


FIGURE 14

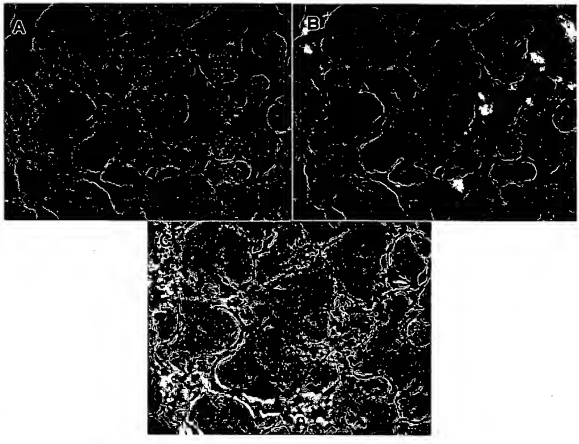


FIGURE 15

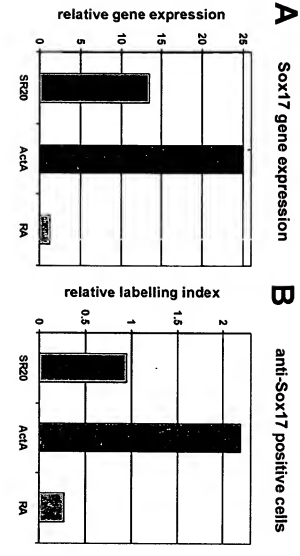

A
Sox17 gene expression
relative gene expression
0
5
10
15
20
25
SR20
ActA
RA
control
B
anti-Sox17 positive cells
relative labelling index
0
0.5
1
1.5
2
SR20
ActA
RA

FIGURE 16

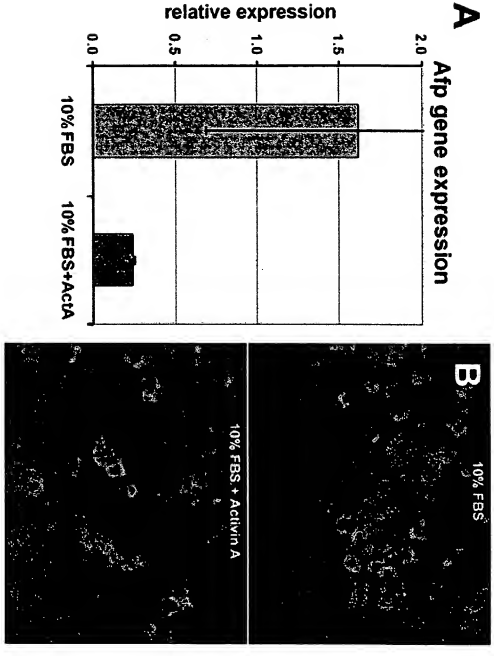

A
Afp gene expression
relative expression
2.0
1.5
1.0
0.5
0.0
10% FBS
10% FBS+ActA
B
10% FBS
10% FBS + Activin A

FIGURE 17

FIGURE 18

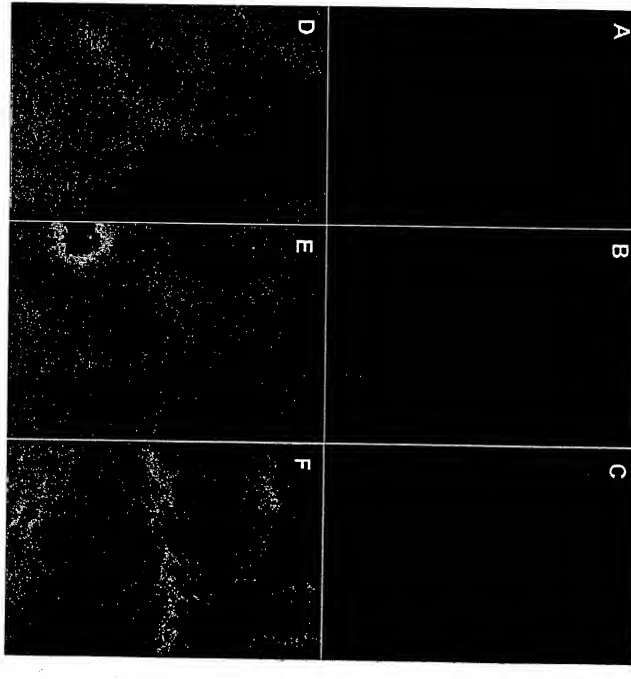


FIGURE 19

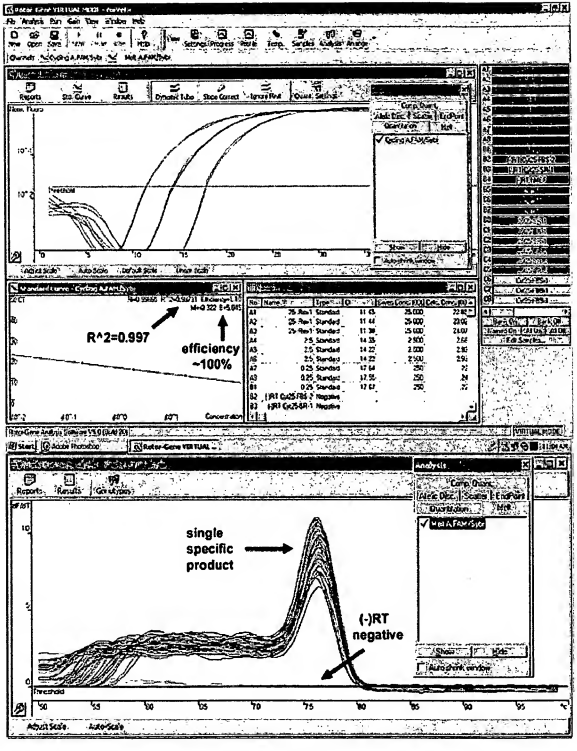

R^2=0.997
efficiency ~100%
single specific product
(-)RT negative

FIGURE 20

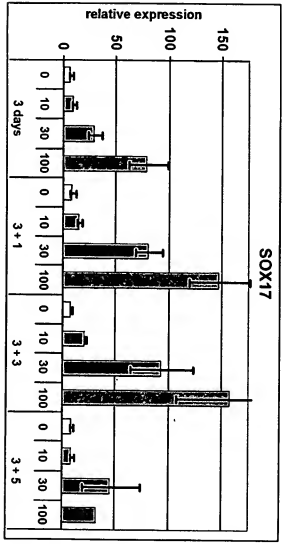

relative expression
0
50
100
150
SOX17
3 days
3+1
3+3
3+5
0
10
30
100

FIGURE 21

FIGURE 22 A

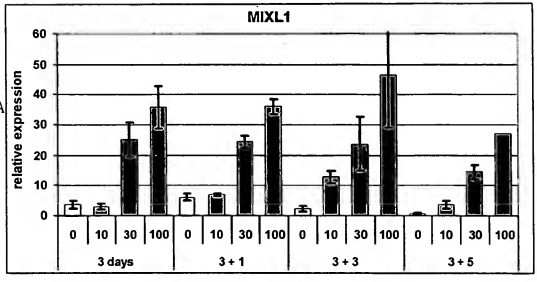


FIGURE 22 B

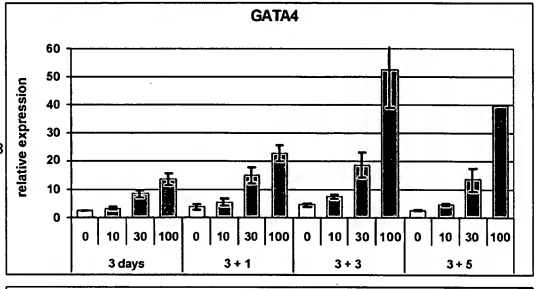


FIGURE 22 C

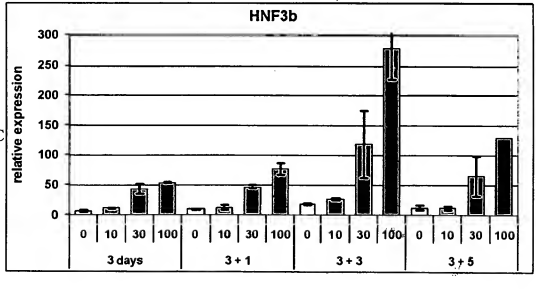


FIGURE 23 A

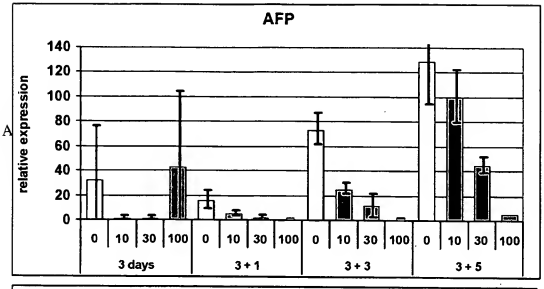


FIGURE 23 B

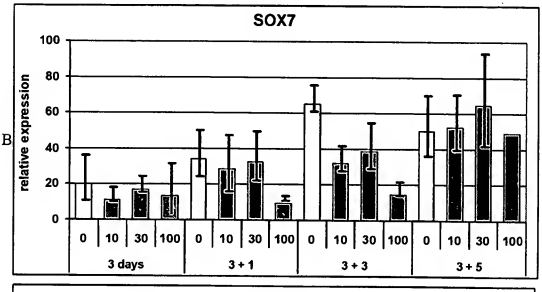
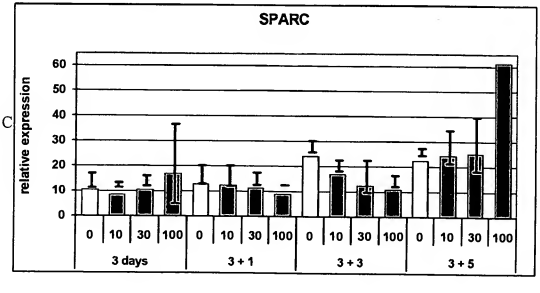


FIGURE 23 C



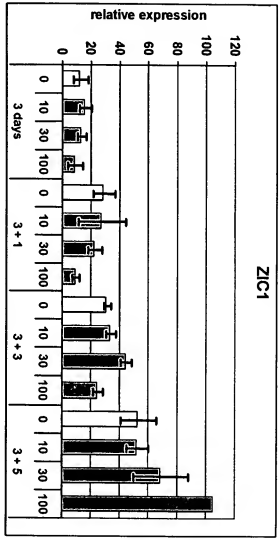


FIGURE 24 A

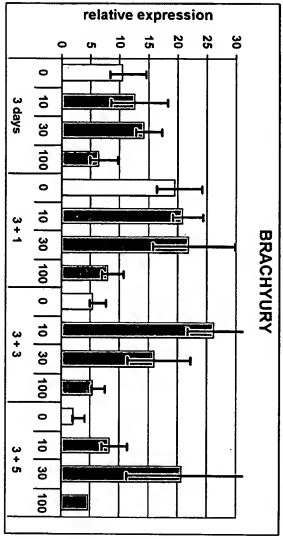


FIGURE 24 B

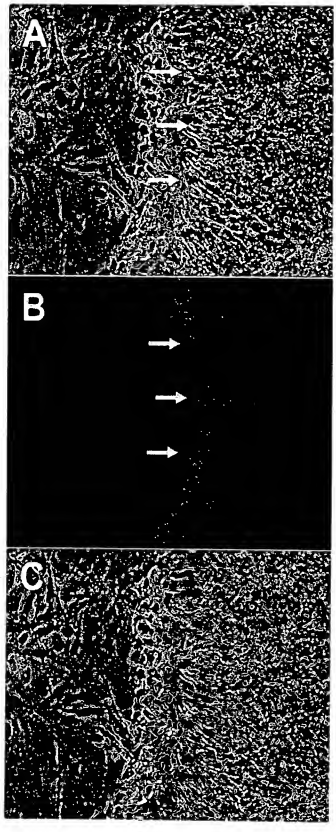


FIGURE 25

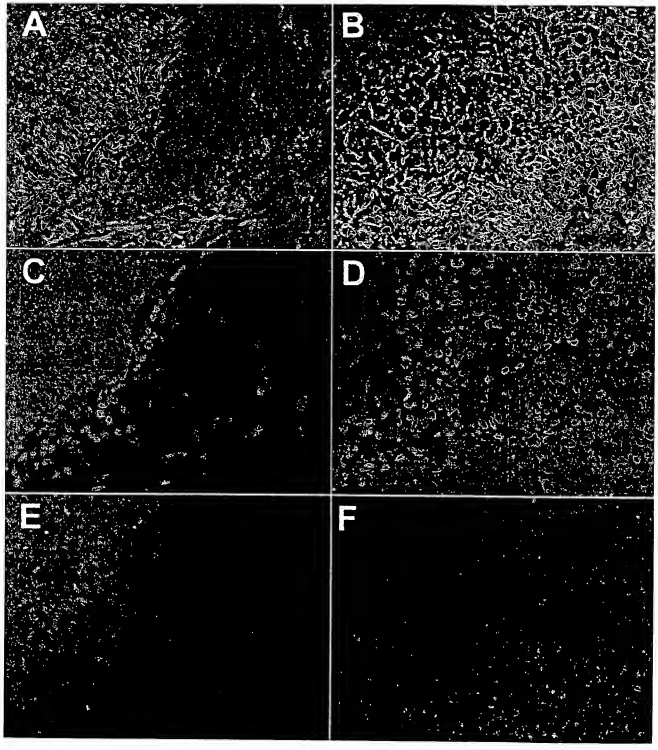


FIGURE 26

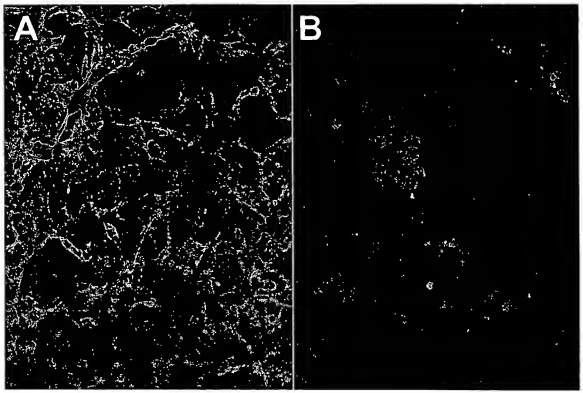


FIGURE 27

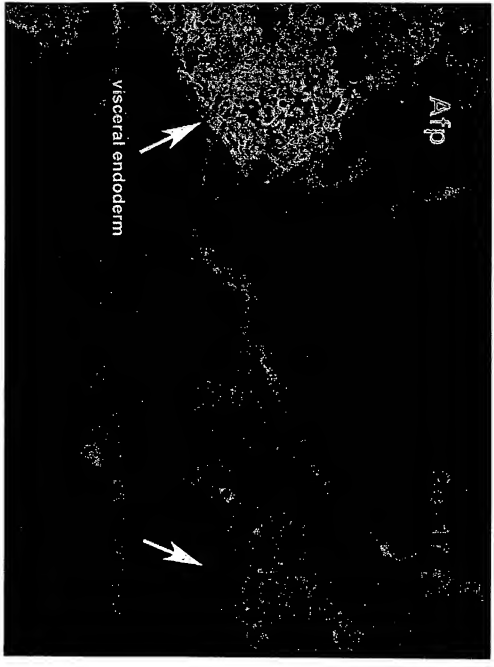


FIGURE 28

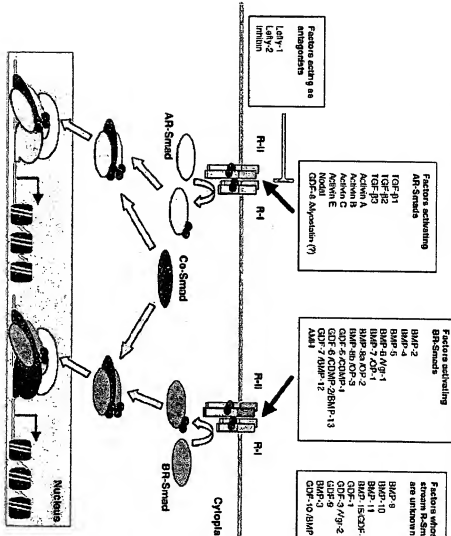

Tgfß family molecules
Factors activating AR-Smads
TGF-ß1
TGF-ß2
TGF-ß3
Activin A
Activin B
Activin C
Activin E
Nodal
GDF-1 Alk-protein (?)
Factors activating BR-Smads
BMP-2
BMP-4
BMP-5
BMP-6/Vgr-1
BMP-7/OP-1
BMP-8/OP-2
BMP-9/GDF-2
GDF-5/CDMP-1
GDF-6/CDMP-2/BMP-13
GDF-7/DMP-12
Factors whose downstream mediators are unknown
BMP-10
BMP-11
BMP-15/GDF-9b
GDF-1/Vg-2
GDF-9
BMP-3
GDF-10/BMP-3b
Factors acting as antagonists
Lefty-1
Lefty-2
inhibin
RI-I
RI-I
RI-II
RI-I
AR-Smad
Co-Smad
BR-Smad
Cytoplasm
Nucleus

FIGURE 29

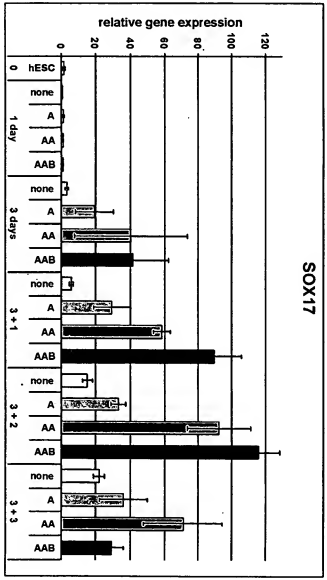

relative gene expression
0
20
40
60
80
100
120
SOX17
0
hESC
1 day
none
A
AA
AAB
3 days
none
A
AA
AAB
3+1
none
A
AA
AAB
3+2
none
A
AA
AAB
3+3
none
A
AA
AAB

FIGURE 30

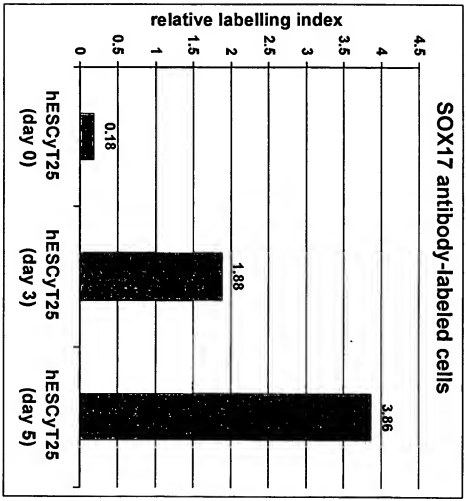

SOX17 antibody-labeled cells
relative labelling index
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
3.86
1.88
0.18
hESCyT25 (day 0)
hESCyT25 (day 3)
hESCyT25 (day 5)

FIGURE 31

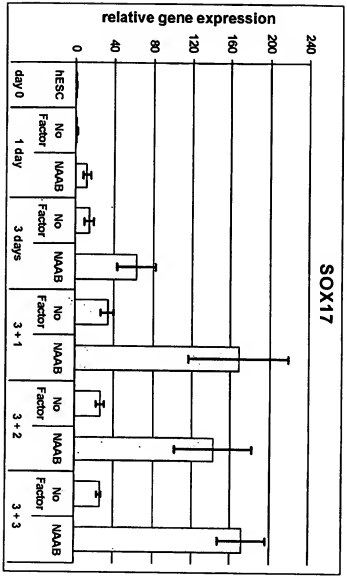

relative gene expression
0
40
80
120
160
200
240
SOX17
hESC
day 0
No Factor
NAAB
1 day
No Factor
NAAB
3 days
No Factor
NAAB
3 + 1
No Factor
NAAB
3 + 2
No Factor
NAAB
3 + 3

FIGURE 32

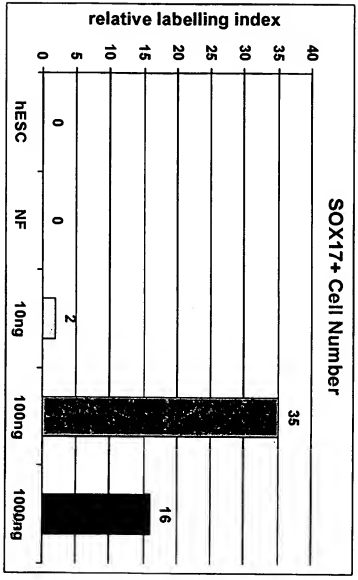

SOX17+ Cell Number
relative labelling index
0
5
10
15
20
25
30
35
40
0
0
2
35
16
hESC
NF
10ng
100ng
1000ng

FIGURE 33

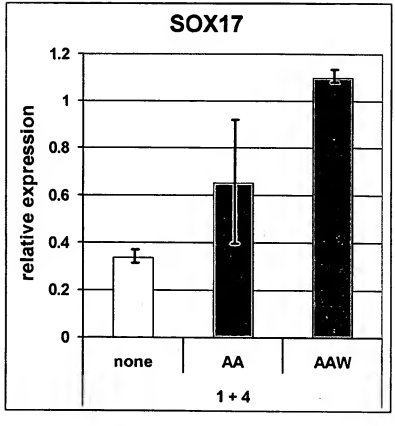


FIGURE 34

FIGURE 35 A

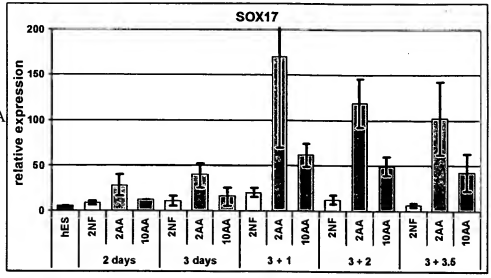


FIGURE 35 B

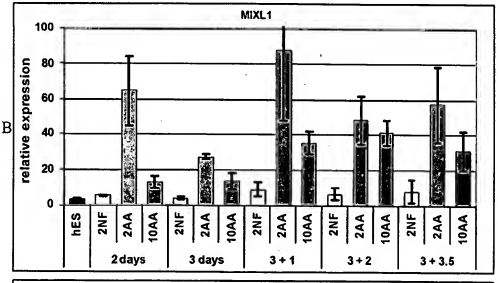
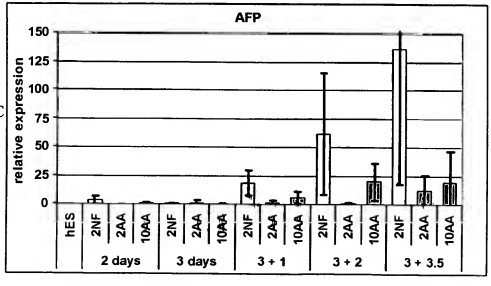


FIGURE 35 C



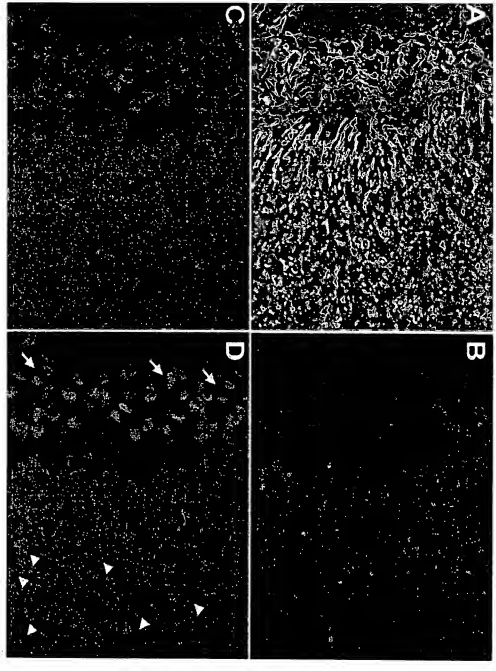


FIGURE 36

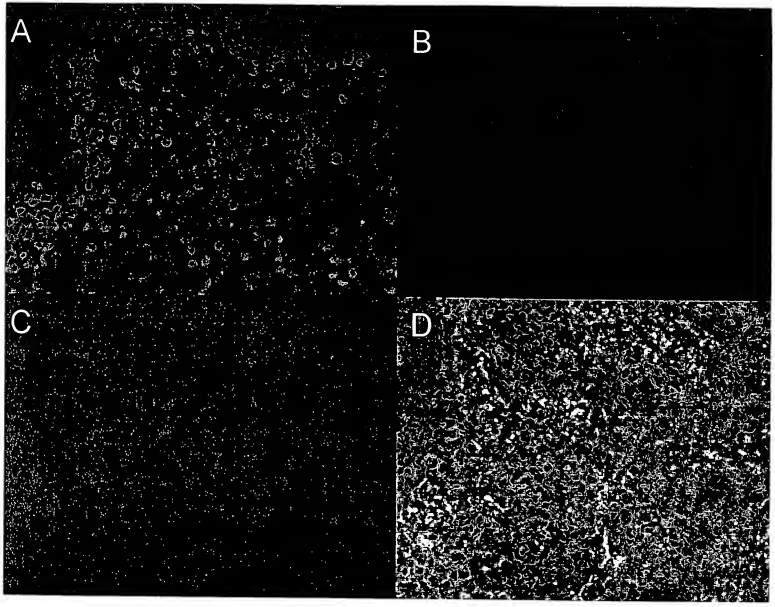


FIGURE 37